THE EXPRESSION, REGULATION AND SELENIUM DEPENDENCE OF THYROIDAL 5'-IODOTHYRONINE DEIODINASE

BY

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DECLARATION OF ORIGINALITY

I declare that the composition of this thesis and the work presented herein is my own. Measurements made by others as part of collaborative studies are indicated in the text.

Stuart Geoffrey Beech

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ABBREVIATIONS

ATP	adenosine 5'-triphosphate
A23187	calcium ionophore, A23187
BSA	bovine serum albumin
Ca^{2+}-PI	calcium-phosphatidylinositol
cAMP	adenosine 3':5'-cyclic monophosphate
CK	creatine kinase
cpm	counts per minute
CPSR-5	control processed serum replacement-5
DIT	diiodotyrosine
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EBS	Earle's balanced salt solution
EDTA	ethylenediaminetetraacetic acid
GDP	guanine diphosphate
GH	growth hormone
GPx	selenium-dependent glutathione peroxidase (classical isoenzyme)
GSH	reduced glutathione
GSSH	oxidised glutathione
GST	glutathione S-transferase
GTP	guanine 5'-triphosphate
ID-I	type-I iodothyronine deiodinase
ID-II	type-II iodothyronine deiodinase

ID-III	type-III iodothyronine deiodinase
IPA	iopanoic acid
KI	potassium iodide
MIT	monoiodotyrosine
M.W	molecular weight
NSB	non-specific binding
PI	phosphatidylinositol
PMA	phorbol 12-mystrate 13-acetate
PTU	6-n-propyl-2-thiouracil
RIA	radioimmunoassay
RNA	ribonucleic acid
ROH	reduced thiol
rT_3	reverse T_3 or 3,3',5'-triiodothyronine
SD	standard deviation
SDS/PAGE	sodium dodecyl sulphate/polyarylamide-gel electrophoresis
Se	selenium
SEM	standard error mean
SP	selenoprotein
TCA	trichloroacetic acid
TEMED	NNN'N-tetramethylethylenediamine
TGA	thymidine-guanine-adenine
TPO	thyroperoxidase
TRH	thyrotropin releasing hormone
Tris	2-amino-2-hydroxymethyl propane-1,3-diol
TSH	thyroid stimulating hormone or thyrotropin
TSIs	thyroid stimulating immunoglobulins

T_3	3,3',5-triiodothyronine
T_4	thyroxine
UGA	uracil-guanine-adenine
3'ut	3'-untranslated
8-BromocAMP	8-Bromo-adenosine 3':5'-cyclic monophosphate
*	denotes a significant difference (p value < 0.05) by a Student's t-test
**	denotes a significant difference (p value < 0.01) by a Student's t-test
***	denotes a significant difference (p value < 0.001) by a Student's t-test

ABSTRACT

Type-I iodothyronine deiodinase (ID-I) is a selenoenzyme which converts thyroxine (T_4) to tri-iodothyronine (T_3), and is found predominantly in liver, kidney and thyroid tissue. The aims of this thesis were : a) to investigate the species variation in thyroidal ID-I expression, b) to study the contribution of ID-I to T_3 production in primary cultures of human and sheep thyrocytes, c) to examine the regulation of ID-I expression by the different 2nd messenger pathways, and d) to study the selenium dependence of ID-I expression, using *in vitro* and *in vivo* models.

Thyroidal ID-I activity varied greatly between species, with the highest activities found in omnivores (rat, man, guinea-pig and mouse), while in herbivores (cattle, sheep, goat, rabbit, pig, llama and deer) thyroidal ID-I was < 0.3% of the activity found in the rat. The livers of all species studied showed similar, and relatively high levels of hepatic ID-I activity.

Primary cultures of sheep and human thyrocytes were established and were shown to produce T_3 in a dose-dependent fashion with respect to thyrotropin (TSH). Using various inhibitors of ID-I activity and *de novo* synthesis, sheep thyrocytes were found to produce T_3 by *de novo* synthesis only. In contrast, human thyrocytes produced T_3 solely by deiodination of endogenous thyroxine (T_4), catalysed by ID-I. In addition, T_3 production was demonstrated to be dependent on iodine supply in sheep but not human thyrocytes. Incorporation of ^{125}I into T_3 and T_4 was only observed with TSH stimulated sheep thyrocytes.

Using human thyrocytes in primary culture I have confirmed that stimulation of ID-I expression by TSH occurred via the cyclic-AMP second messenger cascade. Stimulation of ID-I activity was observed when TSH (0.5 - 1.0 U/L) or 8-Bromo-cAMP (10^{-4} mol/L) was added to the cell medium (3 to 15 fold increase). Addition of TSH at concentrations in excess of 5 U/L or the addition of the phorbol ester, PMA, in conjunction with the calcium

ionophore, A23187 resulted in a decrease in the ID-I activity. Affinity labelling of ID-I with bromoacetyl-[^{125}I]-rT_3 and *in vitro* labelling of ID-I with [^{75}Se]-selenite showed that the reduction in ID-I activity followed a reduction in the amount of the protein, and not a change in the specific activity of ID-I. Addition of PMA/A23187 also prevented the appearance of the TSH-induced "follicles" that were usually observed in monolayer culture.

In human thyrocytes grown in selenium free medium, a small but significant increase in ID-I activity was observed as the extracellular selenium concentration was increased (0 to 1 μM), but this only occurred in the presence of TSH. In the absence of selenium, TSH was still able to induce an increase in ID-I activity. Human thyrocytes were also found to take up and retain [^{75}Se]-selenite in a temperature-dependent manner, suggesting that this is an energy-dependent process. The uptake could not be stimulated by TSH.

The effects of dietary selenium and/or iodine deficiency in rats was also studied. Selenium deficiency caused a decrease in hepatic ID-I activity to approximately 15% of the control value. Iodine deficiency had no significant effect on hepatic ID-I activity. Total thyroidal ID-I activity did not decrease in selenium deficiency, and infact increased in both iodine and combined iodine and selenium deficiencies, with this rise most probably resulting from increased ID-I expression signalled by elevated plasma TSH.

These studies show that : a) thyroidal ID-I expression varies greatly between species, b) human thyrocytes in primary culture utilize ID-I for T_3 production, while sheep thyrocytes rely on *de novo* synthesis, c) thyroidal ID-I expression is under the control of both the cAMP (+) and PI (-) second messenger pathways, and that d) thyroidal ID-I is a selenoprotein protected from selenium deficiency, possibly by an energy-dependent selenium uptake/trapping mechanism.

CONTENTS

Section 1 : INTRODUCTION

1.01 : Anatomy and structure of the thyroid gland

The thyroid gland is a "butterfly" shaped organ located in front of the upper trachea, and consists of two lobes joined together by a small piece of tissue known as the isthmus. The primary role of the thyroid is tosynthesise store and subsequently secrete the thyroid hormones, thyroxine (T_4) and 3,3',5-triiodothyronine (T_3) into the bloodstream. The thyroid gland develops in the human embryo from an invagination in the floor of the primitive pharynx at the level of the first and second branchial arches. This invagination grows downwards from the back of the tongue in front of the primitive pharynx, to form a flask-like vesicle with a narrow neck. This vesicle slowly bifurcates as it continues to grow downwards to its final position in front of the upper trachea. During the descent, the vesicle becomes a solid mass of epithelial cells and loses its connection to the pharyngeal cavity. During atrophy of this connection, small pockets of thyroid epithelial cells can be left along the path taken by the descending thyroid, and hence give rise to functional ectopic thyroid tissue. Before reorganisation of the epithelial cells into follicles, the ultimobranchial body which develops from the fourth branchial pouch, transports calcitonin-producing C cells to the thyroid (Ericson and Fredriksson, 1990).

The basic functional unit of the thyroid is the follicle. The follicle is a spherical, cyst-like structure consisting of a single layer of epithelial cells which encloses the lumen. The lumen contains colloid secreted by the epithelial cells and consists of a glycoprotein-rich solution which is involved in the synthesis and storage of thyroid hormones. The follicles are surrounded by a thin basal lamina, with the extrafollicular spaces containing capillaries, lymph ducts, nerves and C cells. The intercellular spaces in the wall of the follicle are sealed by tight junctions which bind the follicular cells together and form a barrier between the extrafollicular space and the lumen. In addition, the tight junctions

maintain the distinct composition and properties of the apical and the basolateral membranes. The apical membrane has been shown to have microvilli present on its surface, and the presence of peroxidase, aminopeptidase and hydrogen peroxide generating enzymes which are involved in thyroid hormone synthesis. In contrast, the basolateral membrane lacks microvilli and the enzymes involved in thyroid hormone synthesis, but unlike the apical membrane, it contains high levels of Na^+-K^+-ATPase activity and is believed to have Na^+-I^- cotransporter proteins associated with it (Elkholm and Bjorkman, 1990). The differences between the apical and basolateral membranes reflect the differing functions of these membranes in thyroid hormone synthesis and secretion, and will be explained later in this chapter.

In addition to the follicular cells or thyrocytes which are the predominant type of epithelial cells in the thyroid, this organ also contains a small number of C cells. These cells are not involved in thyroid hormone synthesis or secretion but instead produce and secrete calcitonin, a hormone involved in calcium homeostasis.

1.02 : Structure of thyroid hormones

The thyroid is the sole source of thyroxine (T_4) which is the predominant hormone secreted by this endocrine gland. Although T_4 is the most abundant thyroid hormone, the biological actions of thyroid hormones are mediated through 3,3',5-triiodothyronine (T_3), as described below. In addition to T_3 and T_4, there is a further thyroid hormone 3,3',5'-triiodothyronine (rT_3) which has very low biological activity, and is predominantly formed by catabolism of T_4. All three thyroid hormones are iodinated derivatives of two tyrosine residues coupled together by an ether bond, and are also knows as iodothyronines (figure 1a). The phenolic ring of the iodothyronines is regarded as the outer ring, and the iodine positions on this ring are described as either 3' or 5'. The other ring with an alanine side group is regarded as the inner ring, and its iodine positions are designated as 3 or 5.

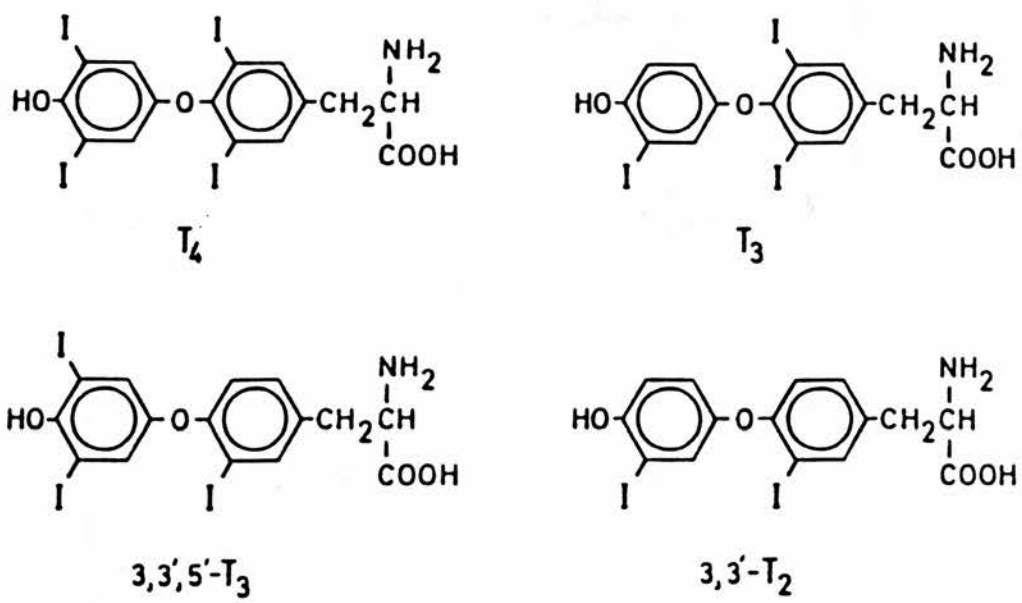


Figure 1a : Structure of iodothyronines.

The numbering of the iodine positions within iodothyronines allows for easier description of the various reactions which thyroid hormones can undergo.

1.03 : Synthesis and secretion of thyroid hormones

The first step in the pathway leading to thyroid hormone secretion is the trapping of iodide within the thyrocyte (figure 1b). Although the basic biochemical mechanism for transport of iodide across the basolateral membrane has not been fully elucidated, the mechanism is believed to be an active process (Wolff, 1964), involving a cotransporter of Na^+ and I^- which transports one I^- ion against its concentration and electrical gradient. This transport is driven by the cotransport of two Na^+ ions into the thyrocyte down the electrochemical gradient of Na^+ (Nakamura *et al,* 1988). Maintenance of this Na^+ electrochemical gradient is provided by the activity of a Na^+- K^+-ATPase and hence the energy to transport iodide is provided indirectly by ATP hydrolysis. Transport of iodide is inhibited by a very large range of compounds which either reduce intracellular ATP concentrations (metabolic inhibitors), inhibit Na^+- K^+-ATPase (cardiac glycosides) or are anions competing for the transporter binding site (halides, pseudohalides, complex anions). Once inside the thyrocyte iodide is not metabolised, but instead leaks across the apical membrane, possibly through iodide channels, to the lumen (Nakamura *et al,* 1988).

The second step in thyroid hormone production is the oxidation of iodide and subsequent iodination of the tyrosyl residues found on thyroglobulin. Thyrogobulin is a very large glycoprotein (molecular weight $\approx$ 660 kDa) secreted by the thyrocyte into the follicle lumen. During the vesicle mediated secretion of this protein, thyroglobulin is glycosylated, phosphorylated and sulphonated on its passage through the rough endoplasmic reticulum and Golgi apparatus (Elkholm and Bjorkman, 1990). Thyroglobulin comprises approximately 80% of the soluble protein fraction in the thyroid, and its tyrosyl residues are the site of iodination. Iodide, once on the luminal side of the apical

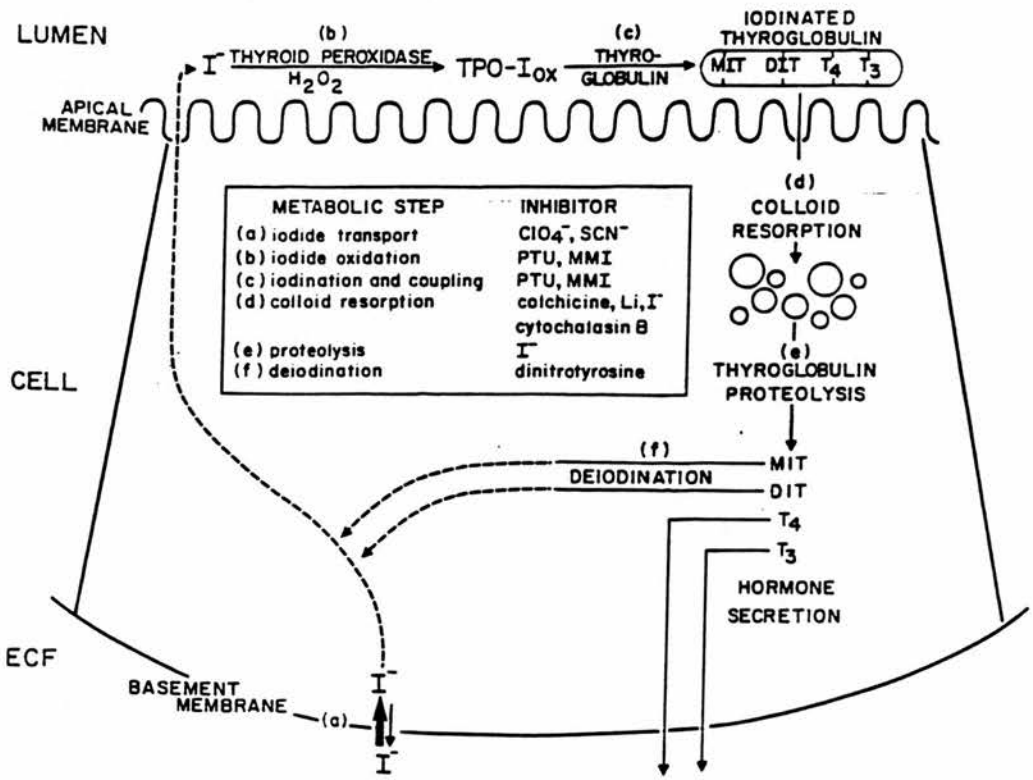


Figure 1b : Diagrammatic representation of the steps in thyroid hormone biosynthesis and secretion.

membrane is oxidized by the haem containing, membrane bound enzyme, thyroperoxidase (TPO) which has a molecular weight of approximately 100 kDa. Oxidation of iodide to the higher oxidation state required for iodination of tyrosine can only be performed by hydrogen peroxide (H_2O_2) or molecular oxygen, as these are the only compounds which have sufficiently high redox potentials to allow this reaction to occur. H_2O_2 has been shown to be produced by the thyroid and hence TPO is believed to use H_2O_2 to oxidize iodide. There has been a great deal of speculation as to the exact mechanism responsible for the production of H_2O_2, with the NADPH-cytochrome c reductase, monoamine oxidase, xanthine oxidase and NADH/NADPH-cytochrome b_5 reductase enzyme systems all being implicated at some time or other (DeGroot and Niepomniszcze, 1977; Yamamoto and DeGroot, 1975; Ohtaki *et al,* 1981; Ohtaki *et al,* 1982). The process is now known to be NADPH-dependent (Virion *et al,* 1984) with two possible mechanisms used. Nakamura *et al* have postulated that an NADPH oxidase produces superoxide ($O_2^{\cdot}$) which is then used by superoxide dismutase to generate H_2O_2 (Nakamura *et al,* 1987). Other workers have proposed that the NADPH oxidase is auto-oxidized by molecular oxygen to form an active species which is capable of producing H_2O_2 (Dupuy *et al,* 1990).

The mechanism by which TPO catalyses oxidation of iodide and subsequently the iodination of tyrosyl residues is also unclear at present. Many alternative mechanisms have been proposed for this iodination, with three proposals receiving the most attention. These involve either free radicals, iodinium ions (I^+) or a hypoiodite intermediate (Nunez and Pommier, 1982; Ohtaki *et al,* 1981; Magnusson *et al,* 1984), and each makes use of H_2O_2 to raise the oxidation state of the free enzyme to form an activated complex (compound I), which is then reduced back to the native state on oxidation of iodide (Bjorkman and Elkholm, 1990; Taurog, 1992). Irrespective of the mechanism used by TPO, tyrosyl residues in thyroglobulin are iodinated using I^- and H_2O_2 to form mono- and diiodotyrosyl residues. These residues are then coupled by TPO within thyroglobulin via

a 3 step reaction, to produce iodothyronines. The iodotyrosyl residues are first oxidized by TPO/H_2O_2 to activated forms which can then couple spontaneously within the same thyroglobulin molecule to produce a quinol ether intermediate. This intermediate then undergoes a structural rearrangement to form an iodothyronine residue and dehydroalanine (Taurog, 1992). The iodothyronine residue formed depends on the two iodotyrosyl residues coupled. Two diiodotyrosyl (DIT) residues when coupled will yield a T_4 residue, while one DIT residue and one monoiodotyrosyl (MIT) residue will couple to yield a T_3 or rT_3 residue, with the MIT residue forming the phenolic or outer ring.

The third step in T_3 and T_4 synthesis is the recovery of iodinated thyroglobulin from the follicle lumen and its subsequent hydrolysis. Iodinated thyroglobulin is believed to be taken back into the thyrocyte by macropinocytosis and micropinocytosis. There is some evidence to suggest that normally iodinated thyrogobulin is selectivity taken up by both of these types of endocytosis in preference to low-iodinated thyroglobulin (Elkholm and Bjorkman, 1990). The thyroglobulin-containing vesicles migrate towards the basolateral membrane, and en route fuse with lysosomes. These lysosomes contain a complex mixture of enzymes (phosphatases, nucleases, proteases, glucoside hydrolases and lipases) generically termed, acid hydrolases since they require an acidic environment for optimum catalytic activity (pH 5). Evidence has been found for an ATP-driven H^+ pump for maintenance of this optimum pH (Fouchier *et al,* 1984). Hydrolysis of thyroglobulin yields iodothyronines (T_4, T_3, rT_3), iodotyrosines (MIT, DIT) and small peptide fragments. Almost all of the MIT and DIT released from thyroglobulin is deiodinated by iodotyrosine deiodinase, an enzyme distinct from ID-I, and the products are then recycled. The iodotyrosine deiodinase has been shown to be a flavoprotein which is capable of deiodinating DIT and MIT, but not T_4 (Rosenberg and Goswami, 1979). This enzyme is found in the mitochondria and microsomes of the thyroid and several peripheral tissues and is NADPH-dependent.

It was originally thought that 5'-deiodination of T_4 to T_3 did not occur in the thyroid, however it is now clear that significant levels of deiodination can occur (Erickson *et al,* 1981; Taurog, 1992). The enzyme responsible for this deiodination of T_4 is the same as that found in liver and kidney, but unlike the liver and kidney forms of ID-I, thyroidal ID-I can be stimulated by the activation of the TSH-receptor (Erickson *et al,* 1982; Ishii *et al,* 1981; Ishii *et al,* 1983; Pazos-Moura *et al,* 1991). This enzyme will be described in detail in a later chapter.

The iodothyronine hormones are believed to passively diffuse out of the thyrocyte, though there may also be a carrier protein involved in this process (Bjorkman and Elkholm, 1990). Once through the basolateral membrane, the iodothyronines enter the general circulation via the capillary blood vessels found in the extrafollicular spaces.

1.04 : Control of thyroid function

Control of thyroid function is exerted by the hypothalamic-pituitary-thyroid axis and has been well described (Reichlin, 1986). In summary, the hypothalamus secretes three neurohormones which are involved in the regulation of thyrotropin (TSH) release from the anterior pituitary. Thyrotropin-releasing hormone (TRH) which is a tripeptide (pyroglutamyl-histidyl-prolinamide) stimulates TSH release from the anterior pituitary, while release of TSH is inhibited by somatostatin (a 14 amino acid peptide) and by dopamine (a monoamine) (figure 1c). As a result of TRH stimulation, the anterior pituitary releases TSH, a 28 kDa glycoprotein consisting of two dissimilar, noncovalently linked subunits (α and β). The α subunit is structurally homologous to the α subunits of follicle stimulating hormone (FSH), luteinizing hormone and human chorionic gonadotrophin (hCG), while the β subunit confers biological activity on TSH when associated with the α subunit. Furthermore, TSH is believed to exist in many different isoforms, which have different relative biological activities. Using a rat thyroid cell line (FRTL-5), acidic forms of TSH

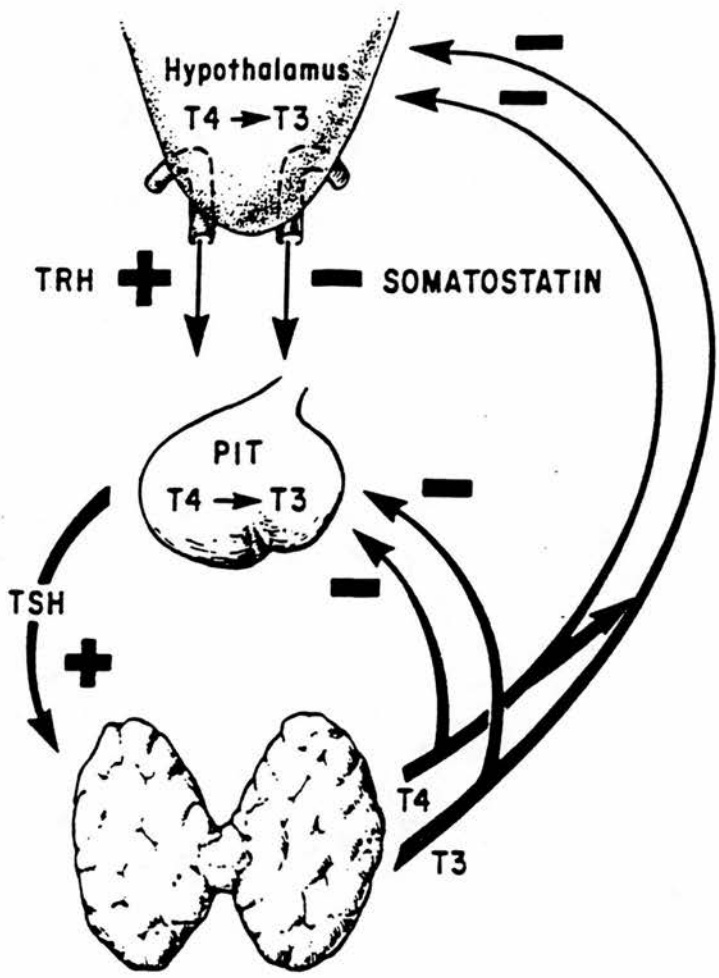


Figure 1c : The hypothalmic-pituitary-thyroid axis (N.B. the action of somatostatin within the diagram is also true of dopamine).

were shown to accumulate more cAMP than alkaline forms, whereas opposite potencies were observed with respect to stimulation of iodide and thymidine uptake (Pickles *et al,* 1992).

TSH release is also influenced by a thyroid hormone feedback mechanism. High concentrations of pituitary T_3 have been shown to reduce both the release and synthesis of TSH, possibly mediated by the increased production or release of an inhibitory factor. This increase in pituitary T_3 can be caused by increased pituitary 5'-monodeiodination of T_4 by type-II 5'-iodothyronine deiodinase or by high plasma T_3 levels. Feedback regulation of TSH release by high levels of thyroid hormones has also been shown to occur at the level of the hypothalamus. High levels of thyroid hormones are thought to induce the release of somatostatin, with no evidence for the inhibition of TRH release (Berelowitz *et al,* 1980).

Historically, TSH was believed to be the sole regulator of thyroid function, with its activity mediated by the cyclic-AMP second messenger cascade (Dumont, 1971). However, it is now thought that the thyroid is also under the control of a rather complicated network of agents including TRH, ATP, bradykinin , dopamine and serotonin, in addition to TSH which is the main regulator of thyroid function (Raspe and Dumont, 1992). TSH and these other regulatory compounds are thought to mediate their effects by activation of specific receptors, which in turn activate one or more of the second messenger systems found in the thyrocyte. Three second messenger systems have been identified in the thyrocyte. These are the cAMP, Ca^{2+}-phosphatidylinositol (Ca^{2+}-PI) and tyrosine kinase cascades. The activation of these cascades will now be summarized :-

The generation of cAMP as a result of hormone action was first described over 25 years ago (Butcher *et al,* 1967) and since than has been well characterised (Birnbaumer *et al,* 1990). The cascade in the thyrocyte is activated when a ligand, typically TSH *in vivo*, binds to and activates its receptor. The activated receptor associates with a

stimulatory GTP-binding transduction protein (G_s) causing the exchange of GDP for GTP, and hence the dissociation and activation of the α subunit of this protein. The activated α subunit activates the catalytic subunit (adenylate cyclase) leading to the production of cAMP from intracellular ATP. Subsequently the activated α subunit returns to its inactive state when the GTP is hydrolysed to GDP by the intrinsic GTPase activity of the α subunit.

As a result of adenylate cyclase activation, an accumulation of intracellular cAMP occurs and this activates the phosphorylation of specific target proteins by protein kinase A, which exists in a number of isoforms. The activity of the cAMP cascade is highly regulated and cAMP is hydrolysed by specific phosphodiesterases which terminate the cAMP signal (Beavo and Reipsynder, 1990). Activation of the cAMP cascade is believed to increase expression of TPO and thyroglobulin (Pratt *et al,* 1989; Pohl *et al,* 1990), with cAMP also stimulating basolateral iodide uptake via a protein synthesis-dependent process (Collison *et al,* 1989). In addition, this cascade is also believed to control secretion of thyroid hormones, most probably by increasing endocytosis of thyroglobulin (Dumont *et al,* 1971).

The existence of the Ca^{2+}-PI second messenger cascade was more recently discovered and described by numerous workers (Michell, 1975; Berridge and Irvine, 1984; Nishizuka, 1984). The initial biochemical reaction of the Ca^{2+}-PI cascade is the hydrolysis of a minor membrane component, phosphatidylinositol 4,5-bisphosphate (PIP_2) to yield diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). This hydrolysis is catalysed by phospholipase C (PLC) which is stimulated by the α subunit of a stimulatory GTP-binding transduction protein (G_q) in much the same way as for adenylate cyclase which is described above. Both DAG and IP_3 can be regarded as second messengers which mediate the effects of the Ca^{2+}-PI cascade. DAG brings about activation of a multifunctional protein kinase (protein kinase C) when in the presence of intracellular Ca^{2+}. IP_3 is known to increase the intracellular Ca^{2+} ion concentration ($[Ca^{2+}]_i$) though release

of Ca^{2+} from an intracellular store which is non-mitochondrial in origin. Increased $[Ca^{2+}]_i$, via activation of calmodulin and other Ca^{2+}-binding proteins and enzymes, elicits a wide range of cellular responses. Activation of the Ca^{2+}-PI cascade is believed to increase apical membrane permeability to iodide (Okajima *et al,* 1988), increase the generation of H_2O_2 (Bjorkman and Ekholm, 1991) and antagonize the cAMP cascade (Decoster *et al,* 1980; Raspe and Dumont, 1992).

Tyrosine kinase activity in cells was first shown 10 years ago (Hunter and Sefton, 1980) and it is now known that the receptors of several growth factors possess tyrosine kinase activity (Ullrich and Schlessinger, 1990). Activation of the tyrosine kinase cascade is initiated when two ligand activated receptors with tyrosine kinase activity form a dimer. This receptor dimer undergoes autophosphorylation of specific tyrosine residues allowing the recognition and phosphorylation of specific substrates to occur (PtdIns 3-kinase, phospholipase $C\gamma$ or ras-GAP) (Ullrich and Schlessinger, 1990; Cantley *et al,* 1991). These proteins all have intracellular signalling capacity which may account for the pleiotropic action of receptors with tyrosine kinase activity.

A number of compounds have be shown to effect the function, growth and differentiation of thyrocytes. TSH, PGE_1, PGE_2, histamine, dopamine, noradrenaline, serotonin and VIP all stimulate the cAMP cascade (Raspe and Dumont, 1992). In contrast, somatostatin causes a reduction in the intracellular concentration of cAMP, presumably by activation of the inhibitory G_i-transduction protein. TRH, bradykinin, ATP and TSH (at approximately a 10-fold higher concentration than required for a maximum cAMP response) have all been shown to stimulate the Ca^{2+}-PI cascade (Chanoine *et al,* 1993; Raspe *et al,* 1991). Although direct evidence has proved elusive, various workers have postulated that EGF, IGF_1 and insulin modulate thyrocyte growth via the intrinsic tyrosine kinase activity of their receptors (Contor *et al,* 1988; Formisano *et al,* 1991; Takahashi *et al,* 1991). In a recent review, Raspe and Dumont summarized data from several workers

and postulated the function of each cascade in thyrocyte regulation (Raspe and Dumont, 1992). It was argued that cAMP, which is mainly activated by TSH, controls the differentiation, proliferation and the secretion of thyroid hormones. In contrast, activation of the Ca^{2+}-PI cascade by neurotransmitters and high levels of TSH was argued to control thyroid hormone synthesis by regulating of the supply of H_2O_2 and I^- to TPO. Activation of the Ca^{2+}-PI cascade also reduces intracellular cAMP levels, thereby antagonizing the action of agents which elevate intracellular cAMP concentration. Finally, the action of EGF, IGF_1 and insulin, which can be classified as growth factors, and was postulated to occur via the tyrosine kinase cascade, thereby modulating thyrocyte growth while causing dedifferentiation.

1.05 : Transport and the biological role of thyroid hormones

Transport of thyroid hormones from the thyroid to the target organs in plasma predominantly involves their binding to a number of plasma transport proteins. These transport proteins vary widely in their plasma concentration and affinity for the thyroid hormones. The main advantage of having the bulk of thyroid hormones bound to plasma proteins is to prevent excessive loss into the urine, while the hormones are still readily available if required. There are three plasma proteins which are responsible for binding thyroid hormones. The first of these proteins is thyroxine-binding globulin (TBG) which has a molecular weight of 54 kDa. This α_2 globulin binds 70% of plasma T_4, 80% of plasma T_3 and consists of a single polypeptide chain with 4 complex polysaccharide units on its surface. TBG is present in the plasma at a concentration of approximately 3.6×10^{-7} mol/L, and has one binding site per molecule. In contrast thyroxine-binding prealbumin (TBPA) is tetrameric, with 2 binding sites and a plasma concentration of approximately 4.6×10^{-6} mol/L. TBPA also has a molecular weight of 54 kDa, and binds 20% of T_4 and 10% of T_3. Finally, the monomeric protein albumin, with its 7 binding sites and molecular

weight of 66 kDa, binds approximately 10% of both T_4 and T_3. These three proteins bind virtually all the T_4 and T_3 found in the plasma, leaving less than 0.05% of T_4 and 0.5% of T_3 circulating as free hormones (Robbins and Edelhoch, 1986).

Entry of thyroid hormones into cells has previously been regarded as a passive diffusion process. However, there is considerable evidence for the active transport of thyroid hormones, with endocytosis and a Na^+ ion facilitated transporter both implicated. Once inside the target cell both T_4 and T_3 can bind to a number of soluble proteins, the identity of which varies between tissues. Of the cytosol thyronine-binding proteins (CTBP) which have been found, so far only a few have been identified, and these include haemoglobin, myoglobin, glutathione-S-transferases (GSTs) and pyruvate kinase (Davis, 1992). The effect of T_4 and T_3 on these proteins is not fully characterized, though the activities of both pyruvate kinase and the GSTs are inhibited by these thyroid hormones. Intracellular T_4 has also been shown to cause an increased degradation of type-II iodothyronine deiodinase via the rearrangement of f-actin, a major component of the cytoskeleton (Safran *et al,* 1993).

The main sites of T_3 action are the mitochondria and the nucleus. There is considerable evidence to support an effect of T_3 on the mitochondria, with 3 possible site of action implicated (Davis, 1992). The first of these is the inner mitochondrial membrane protein, ADP - ATP translocase. This protein has been shown to bind a significant amount of T_3, and is known to transport ADP into the mitochondria in exchange for ATP. Regulation of ATP synthase is also believed to be under the control of T_3, with T_3 increasing the activity of this ATP generating enzyme. Finally, the expression of various components of the electron transport chain are also increased by T_3. By stimulation of these 3 components, T_3 can bring about increased ATP production by increasing oxidative phosphorylation.

The ability of T_3 to bind to a nuclear receptor has been known for some time. However in the last 10 years the nature of this interaction has been described in more detail. The current state of knowledge has been summarised in a recent review (Oppenheimer, 1992). To simplify this further, the main elements which confer the actions of T_3 are coded for by two genes of the c-*erb* A superfamily of genes, which are classified as c-*erb* A-α and c-*erb* A-β. As a result of alternate splicing, the c-*erb* A-α gene gives rise to mRNA for 3 distinct proteins (α_1, α_2 and rev-*erb* A). The α_1 product is known to bind T_3 and exhibit similar binding characteristics as T_3 receptors isolated from nuclei. The α_2 and rev-*erb* A products do not bind T_3 and are believed to be involved in the regulation of gene expression by competing with the active receptors for the sites of receptor-DNA interaction. The c-*erb* A-β gene gives rise to 2 distinct mRNA products, β_1 and β_2, the protein products of which bind T_3. The expression of these 5 proteins varies widely between species and tissues, though in general, the α_1 and β_1 are believed to bind T_3 and mediate the appropriate changes in gene expression. The T_3-receptor complex is believed to bind to a gene specific sequence of nucleotides, upstream from the transcription start site. These nucleotides sequences are termed, thyroid response elements (TRE). The TRE for the growth hormone (GH) gene is known to lie between nucleotide residues, - 190 and - 173. Interaction of the T_3-receptor complex is believed to involve "zinc fingers", with evidence in support of the T_3-receptor complex acting in a heterodimeric form. The interaction of the T_3-receptor complex with the TRE is believed to increase transcription of the gene, and hence increases mRNA formation for the appropriate specific protein.

1.06 : Metabolism of thyroid hormones

Thyroid hormones (iodothyronines) can undergo various reactions classified as either 5'-monodeiodination, 5-monodeiodination, conjugation, decarboxylation, deamination or

ether bond cleavage. The importance and occurrence of these various reactions are described below and summarised for T_4 in figure 1d.

a) Ether bond cleavage, decarboxylation and deamination

These three reactions are the least important in the metabolism of iodothyronines (Visser, 1990). Ether bond cleavage is an oxidative reaction which occurs when tissue peroxidase activity is stimulated, and yields diiodotyrosine as one of its products. This reaction is believed to occur in phagocytosing leucocytes, and may be more important in the rat than in man.

Decarboxylation and deamination are side chain modification reactions both of which can occur in man. Decarboxylation and deamination of T_4 account for only 2% of T_4 metabolism forming 3,3',5,5'-tetraiodothyroacetic acid (TA_4). In comparison, decarboxylation and deamination of T_3 accounts for about 14% of T_3 metabolism, forming 3,3',5-triiodothyroacetic acid (TA_3).

b) Conjugation reactions of iodothyronines

Conjugation of compounds is a major metabolic reaction for the disposal of a large variety of synthetic and endogenous compounds (Visser, 1990). Iodothyronines are no exception, and can be deactivated by conjugation with glucuronic acid or sulphate. Both of these processes occur predominantly in the liver and increase the water solubility of the iodothyronines, hence favouring excretion in the urine or bile.

Glucuronation of iodothyronines, requires uridine diphosphate (UDP)-glucuronic acid (UDPGA) which is formed from glucose-1-phosphate and uridine triphosphate (UTP) via an intermediate (UDP-glucose). The conjugation of UDPGA to the OH-group of the iodothyronine outer ring requires the catalytic activity of UDP-glucuronyltransferases (UDPGTs), a group of homologous, transmembrane enzymes found in the liver, kidney,

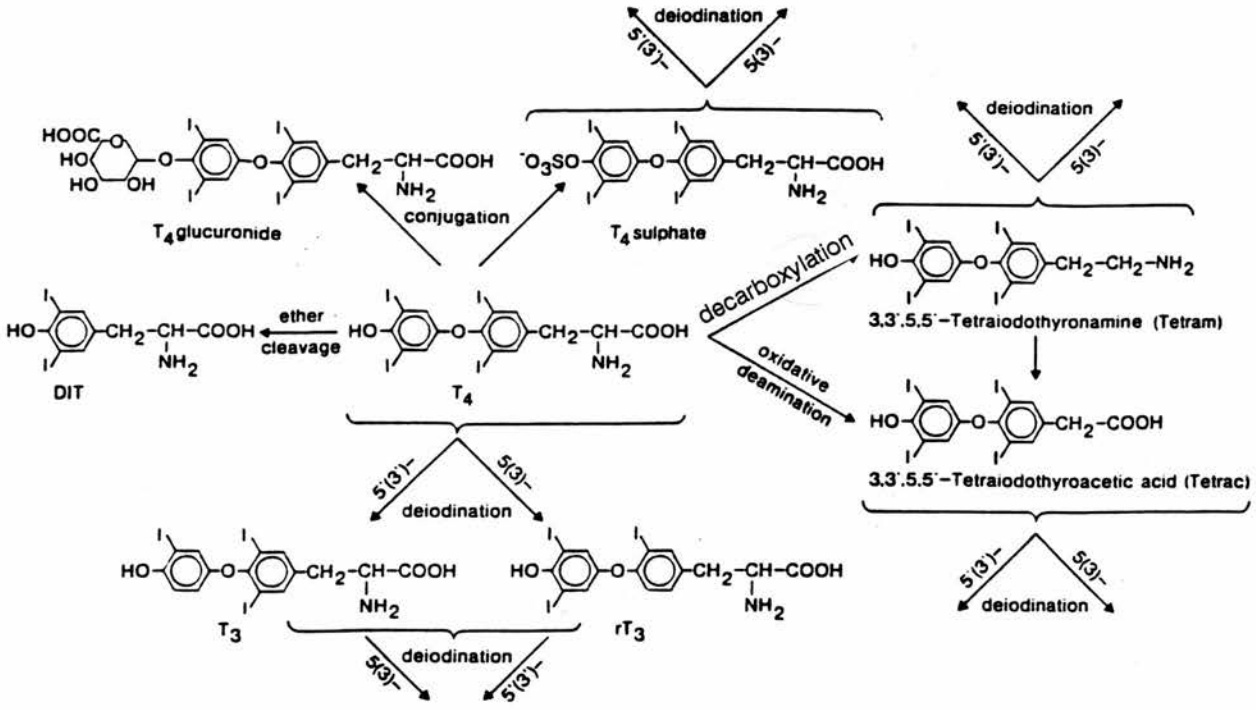


Figure 1d : Pathways for the metabolism of T_4.

intestine and numerous other tissues. The products of this conjugation are UDP (which is recycled), and iodothyronine-glucuronide conjugates. All glucuronide conjugates excreted in the bile can undergo enterohepatic cycling, and iodothyronines are no exception. The action of β-glucuronidase found in the intestine allows hydrolysis of iodothyronine-glucuronide conjugates and hence the iodothyronines can once again enter the circulation.

Sulphation of phenolic compounds including iodothyronines is catalysed by a homologous group of enzymes known as phenol sulphotransferases (PSTs) which are found in various tissues including the liver, kidney, intestine and brain. Sulphation of the 4'-OH group of iodothyronines is believed to be catalysed by the TS-type PST, using 3',-phosphoadenosine-5'-phosphosulphate (PAPS) as the sulphate donor, to yield the iodothyronine-sulphate conjugate. The metabolic pathways of iodothyronine-sulphate conjugates are described in the next section.

c) Deiodination of iodothyronines

The deiodination of iodothyronines plays a central and very important role in the production of active thyroid hormone and the subsequent catabolism of the iodothyronines. In the rat, approximately 80% of plasma T_3 is normally derived from 5'-monodeiodination of T_4, with the remaining 20% being secreted directly by the thyroid (Visser, 1988). This 5'-deiodination of T_4 occurs predominantly in peripheral tissues such as liver and kidney, and is catalysed by type-I iodothyronine deiodinase (ID-I). In addition within certain tissues, a second deiodinase (type-II iodothyronine deiodinase, ID-II) is found which can catalyse 5'-deiodination of T_4. However unlike T_3 produced by ID-I, T_3 produced by ID-II is not thought to be available to the general circulation and is utilised locally (Silva and Larsen, 1983). Both 5 and 5'-deiodination can also deactivate thyroid hormones by deiodination to form thyromimetically inert metabolites. Thyroxine undergoes

5-monodeiodination to produce the inactive form of thyroid hormone, rT_3. This reaction can be catalysed by ID-I or by a third deiodinase, type-III iodothyronine deiodinase (ID-III). The rT_3 produced by ID-I or ID-II can undergo 5'-deiodination to produce diiodothyronine (T_2), which can also be produced by 5-deiodination of T_3. The T_2 produced by both pathways is then subjected to further deiodination to produce 3' or 3-monoiodothyronine.

The sulphate conjugates of T_2, T_3 and T_4 have all been shown to have lower K_m values than their unconjugated analogues for deiodination by the type-I iodothyronine deiodinase (ID-I). The outer ring deiodination (5'-deiodination) of T_2 has been shown to increase as a result of sulphation. In contrast, only inner ring deiodination (5-deiodination) of T_3S and T_4S by ID-I is increased, with no outer ring deiodination of T_3S or T_4S detected. Sulphation of rT_3 has no effect on either 5 or 5'-deiodination by ID-I. Hence in general, sulphonation of iodothyronines results in more rapid deiodination of iodothyronines within the liver to the thyromimetically inactive forms, 3' or 3-monoiodothyronine (Visser, 1990).

1.07 : Selenium and selenoproteins

The trace element, selenium is found in the 6th group of the periodic table, one row below sulphur, and has an atomic weight of 78.96. Selenium and sulphur share similar chemical properties, such as covalent radii and the ability to make use of $d\pi$-$p\pi$ multiple bonding. As selenium and sulphur are similar, compounds that contain carbon-selenium-carbon bonds can be metabolised by the enzyme which catalyses the reaction of the corresponding sulphur compound (Sunde, 1990).

Selenium was first recognised as an essential nutritional element in 1957 when it was shown to prevent liver necrosis in vitamin E-deficient rats (Schwarz and Foltz, 1957). Subsequently, glutathione peroxidase (GPx) was identified as a "functional" selenoprotein (Rotruck *et al,* 1973) and consequently the action of GPx was used to explain the synergistic effects of selenium and vitamin E supplementation in the prevention of cardiac

and skeletal muscle myopathies, and liver necrosis observed in farm and laboratory animals (Combs and Combs, 1986b). Other selenium-binding proteins or protein subunits have now been demonstrated in bacteria and in animals (Evenson and Sunde, 1988; Behne *et al,* 1988). Selenium can be incorporated into proteins as a selenomethionine (non-specifically instead of methionine) or as selenocysteine (specifically in response to a codon UGA as described below) (McConnell and Hoffman, 1972; Voordouw *et al,* 1989; Chambers *et al,* 1986; Mullenbach *et al,* 1987). To date, only 6 mammalian selenoproteins have been identified which have selenium specifically incorporated into the protein as a selenocysteine residue :-

Cytosolic glutathione peroxidase (GPx) or "classical" GPx, which was first described by Rotruck *et al*, consists of 4 identical subunits, each with a molecular weight of 23 kDa and one selenocysteine residue (Rotruck *et al,* 1973).

Phospholipid hydroperoxide glutathione peroxidase (PLGPx) which is found in porcine heart and liver has been shown to be a 20 - 23 kDa monomeric selenoenzyme, distinct from "classical" GPx (Ursini *et al,* 1982; Schuckelt *et al,* 1991). Unlike "classical" GPx, PLGPx can metabolise phospholipid hydroperoxides, lipid peroxides and low density lipoprotein, and is believed to prevent peroxidative damage to membranes (Thomas *et al,* 1990; Maiorino *et al,* 1991).

Plasma glutathione peroxidase (PGPx) is also distinct from the "classical" GPx and is tetrameric with a molecular weight of 21.5 - 23 kDa per subunit (Takahashi and Cohen, 1986; Takahashi *et al,* 1990). PGPx is a glycoprotein, and is believed to be synthesised almost exclusively in the kidney (Yoshimura *et al,* 1991). The function of PGPx may lie in the metabolism of GSH in the kidney, and hence the protection of the membranes which filter the blood to produce urine.

Selenoprotein P is a plasma selenoprotein with a molecular weight of 55 kDa (Burk and Gregory, 1982) and has been shown to be a glycoprotein containing 7.5

selenocysteinyl, 17 cysteinyl and 23 histidyl residues per mole (Read *et al,* 1990). Cloning of the protein has shown 10 inframe TGA codons which could specify selenocysteine (Hill *et al,* 1991)}. The role of selenoprotein P is yet to be established though selenium transport and antioxidant roles have been proposed.

Type-I iodothyronine 5'-deiodinase (ID-I) has been demonstrated to be a selenoenzyme, with a UGA codon present in the mRNA for the enzyme (Berry *et al,* 1991b). A full description of ID-I and its role in thyroid hormone metabolism can be found later in this introduction.

Selenoprotein W is the most recent selenoprotein to be identified in skeletal muscle (Vendeland *et al,* 1993), though its existence has previously been suggested by studies into white muscle disease found in selenium deficient animals (Muth *et al,* 1959). Four proteins were found by Vendeland *et al* with molecular weights of 9550, 9596, 9858 and 9898 daltons, with one selenium atom per mole of protein. At present there is no information as to the exact structure of the native protein or its role.

The carbon for selenocysteine synthesis is known to come from serine (Sunde and Evenson, 1987), with the synthesis of specific-selenoproteins shown in bacteria to be dependent on the products of 4 genes (SelA, SelB, SelC and SelD) (Bock *et al,* 1991b; Burk, 1991). The SelC gene product is a unique form of tRNA, which acts as a serine/phosphoserine carrier and recognises the UGA stop codon. The SelA gene codes for a pyridoxal phosphate-containing, selenocysteine synthetase enzyme which converts the serine on the tRNA, specified by the SelC product to selenocysteine in the presence of the product form by the SelD protein, which is chemically equivalent to selenide. The SelD product requires ATP and magnesium for its activity, and is thought to produce phosphoselenoate (Ehrenreich *et al,* 1992). The serine is first phosphorylated to phosphoserine before the phosphate group is substituted with selenite, as phosphoselenoate to produce selenocysteinyl tRNA. The SelB product is required for

cotranslation and insertion of selenocysteine into proteins and is similar to the elongation factor Tu which transports amino acid tRNAs to the ribosome, however SelB is believed to be selenocysteinyl tRNA specific. The gene products for selenocysteine incorporation in eukaryotes and prokaryotes are believed to be very similar (Mizutani *et al,* 1992).

In addition to a UGA codon, a specific 3'-untranslated (3'ut) segment needs to be present for human and rat ID-I expression (Berry *et al,* 1991a). A different 3'-ut sequence is required for incorporation of selenocysteine into "classical" GPx, however the 3'ut sequences of ID-I and GPx can be interchanged, without loss of expression. These 3'ut sequences are believed to form a 3-dimensional "stem loop" structure, which allows recognition of the UGA codon as selenocysteine instead of a termination codon. Rat mRNA for selenoprotein P has also been shown recently to possess "stem loop" structures in the 3'ut sequence which are required for translation (Berry *et al,* 1993). In contrast, the "stem loop" structure which confers selenocysteine incorporation in bacterial formate dehydrogenase is not in the 3'ut sequence, but in the coding region (Bock *et al,* 1991a; Heider *et al,* 1992).

1.08 : Location and properties of iodothyronine deiodinases

There are 3 known isoenzymes of iodothyronine deiodinase, and these have been given the names type-I, II and III. Although these isoenzymes have not been purified the enzymes have been characterised on the basis of a number of physicochemical properties and are summarised below, in table 1a and also in a number of reviews. (Kaplan, 1986; Leonard and Visser, 1986; Silva and Larsen, 1986; Visser, 1989; Kohrle *et al,* 1993; Leonard, 1990; Berry and Larsen, 1992).

	Type I	Type II	Type III
Deiodination site	5 and 5'	5'	5
Substrate preference	rT3>T4>T3	T4>rT3	T3>T4
Reaction catalysed	T4 to T3 T3 to T2 rT3 to T2	T4 to T3 rT3 to T2	T4 to rT3 T3 to T2
Tissue location	Kidney, Liver Thyroid, CNS Pituitary	CNS,BAT, Pituitary	CNS, Placenta Skin
Role	Provides plasma T3	Local T3 Production (BAT - plasma T3)	Uncertain
Propylthiouracil	Inhibited	No effect	No effect
Iopanoic acid	Inhibited	Inhibited	Inhibited
Selenoenzyme	Yes	No	Probably not
Subunit Molecular Mass	27-kd	29-kd	Not known
Hypothyroidism	Liver, Kidney decreased. Thyroid increased	Increased	Decreased
Hyperthyroidism	Increase	Decrease	Increase
Low-T3 Syndrome	Decreased	No change	No change
Selenium deficiency	Liver, Kidney decreased. Thyroid increased	Decreased	Not known
Iodine deficiency	Liver, Kidney no change. Thyroid increased	Pituitary no change BAT increased Brain increased	Not known

Table 1a : Properties of the iodothyronine deiodinases.

a) Type-I iodothyronine deiodinase (ID-I)

ID-I has been shown to catalyse both 5 and 5'-monodeiodination and exhibits a clear order of substrate preference, with rT_3 deiodinated in preference to T_4 and T_3 ($rT_3 > T_4 > T_3$). This enzyme is localised in the microsomal membrane where it represents 0.01 - 0.025% of membrane-associated protein. ID-I is believed to consist of two subunits with a total molecular weight of 50 - 60 kDa, although it is unclear if the enzyme is homo- or heterodimeric. The substrate binding subunit of ID-I has been shown using ^{125}I-bromoacetyl derivatives of rT_3, T_4 and T_3 to have a molecular weight of 27 - 28 kDa. ID-I catalyses both 5 and 5'-deiodination with the pH and level of substrate sulphation determining which reaction predominates. The optimum pH for 5-deiodination is 8.0 - 8.5, with optimum 5'-deiodination occurring at pH 6.5 - 7.0. Sulphation of the 4'-OH of T_3 and T_4 has been shown to enhance 5-deiodination, while decreasing 5'-deiodination. Therefore a combination of pH change and sulphation may be responsible for determining whether ID-I primarily catalyses 5 or 5'-deiodination. At present it is unclear if 5 and 5'-deiodination by ID-I is catalysed by the same active site. However, the reaction kinetics and inhibitors of 5-deiodination are identical to those for 5'-deiodination, and hence this is consistent with the use of the same site by both reactions, with some slight change or "wobble" in the active site.

It has been proposed that the rate of deiodination by ID-I is controlled by either an NADPH-glutathione-redox system or an NADPH-thioredoxin-dependent redox system. The activity of ID-I has been shown to be dependent on the presence of reduced glutathione (GSH). The ratio of reduced GSH to oxidised GSH (GSH : GSSG) appears to affect the rate of deiodination, with the absolute concentration of GSH having no significant effect. Data from *in vivo* experiments suggests that the redox state of the cell, presumably via the ratio of GSH : GSSG, controls ID-I activity. Hence, NADPH-linked reduction of GSSG by GSH reductase may well be the important physiological control system. However, there

is also *in vitro* evidence for a GSH-independent supply of reducing equivalents to ID-I by a NADPH-thioredoxin coupled system. Whether a similar system is present *in vivo* is unclear. It is of interest that *in vitro* assays of ID-I activity usually make use of dithiothreitol (DTT), which is a more potent activator of ID-I than GSH.

The reactions of ID-I have been shown to follow "ping-pong-type" kinetics. Steady-state reaction kinetics for ID-I have shown that the reaction consists of two steps, with reactivation of the native enzyme requiring reduced thiols (RSH) (figure 1e). The active, reduced form of the enzyme (E-Se$^-$) reduces the iodine-carbon bond of the iodothyronine by the transfer of 2 electrons, with the Se$^-$ of the enzyme forming a selenoyl iodide with the newly released iodide. This partially oxidised group is then reduced by reduced thiols to regenerate the activated enzyme. One of the important characteristics of ID-I is its inhibition by 6-n-propyl-2-thiouracil (PTU) at micromolar concentrations; with the reaction scheme described for ID-I elucidated with the use of this compound (figure 1e). PTU is believed to reversibly form a stable compound with the oxidised form of the enzyme. The activity of ID-I can also be inhibited by propranolol, gold thioglucose and by iodinated radiocontrast agents (ipodate and iopanoic acid). The mechanism of action for propranolol remains to be elucidated, while the competitive inhibition by radiocontrast agents may rest in the iodine content of this group of compounds. A mechanism of action for gold thioglucose has been suggested (Berry *et al,* 1991c), and involves the formation of a stable complex when gold reversibly binds to the reduced form of the enzyme (figure 1e).

The first evidence that selenium was required for the function of ID-I came from a series of comprehensive studies using selenium deficient rats. These experiments have been comprehensively reviewed recently (Beckett and Arthur, 1993). The first suggestion that ID-I was a selenoenzyme came when selenium status was observed to affect plasma T_4 and T_3 concentrations. Selenium deficiency caused significant increases in plasma

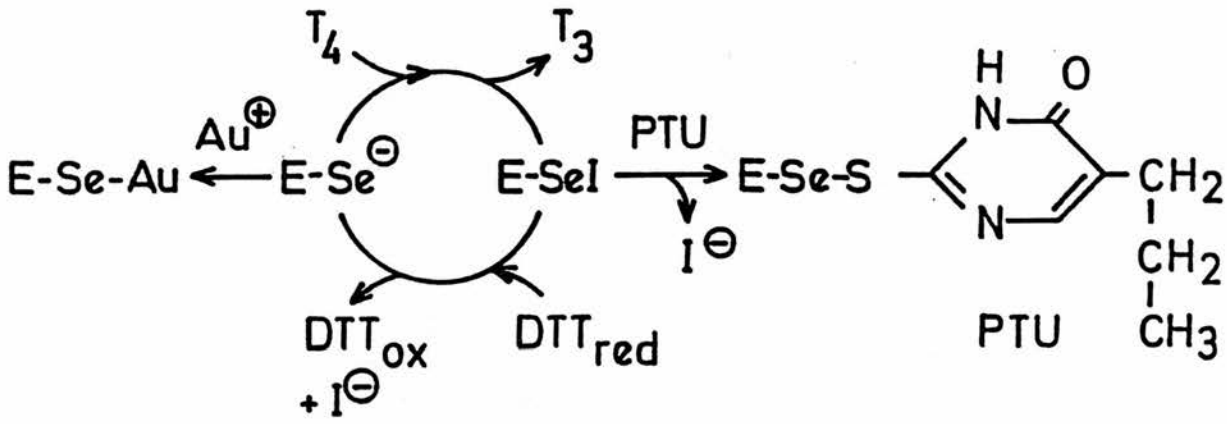


Figure 1e : Proposed mechanism of Type-I iodothyronine deiodinase and its inhibition by PTU and gold thioglucose.

total and free T_4 concentrations, and was accompanied by a reduction in plasma total T_3. ID-I activity in the liver and kidney homogenates was markedly impaired in selenium deficiency, and could not be restored by adding thiols or pyridine nucleotides. The described changes occurred within four weeks, and ID-I activity could be restored within 3 days by a single, 200μg intraperitoneal injection of selenite. The decreased ID-I activity was shown not to be due to reduced food intake, increased oxidative damage, lack of essential co-factors or the presence of an ID-I inhibitor. This evidence lead to the suggestion that ID-I was a selenoenzyme.

To confirm this hypothesis, ^{75}Se-selenite was administered to rats, and this resulted in the substrate binding subunit of ID-I being labelled with ^{75}Se (Arthur *et al,* 1990b). Other workers independently produced similar ^{75}Se labelling of ID-I using the rat as a model and subsequently showed that astoiochiometricamount of selenium is associated with the ID-I substrate binding subunit (Behne *et al,* 1990). In addition, affinity labelling experiments using [^{125}I]-bromoacetyl derivatives of rT_3, T_3 and T_4 confirmed that the fall in ID-I activity observed in selenium deficiency was due to decreased expression of ID-I and not a modification of enzymic activity (Arthur *et al,* 1991b; Beckett *et al,* 1992).

Whilst the above experiments were being reported, Berry and Larsen were using expression cloning in Xenopus oocytes to characterise rat ID-I. The full details of these experiments and subsequent work can be found in a recent review (Berry and Larsen, 1992). In summary, rats were made hyperthyroid by administration of 5 daily injections of T_3. Subsequently, Poly A + mRNA was prepared from hepatic tissue using standard methods and reverse transcribed using an Xba 1 oligo dT primer. The cDNA was size-selected for sequences between 1.8 and 2.6 kb, ligated to EcoR1 adaptors and then inserted in the phage vector λ Zap II. The resulting library was subdivided, amplified and converted to a blueprint plasmid by *in vivo* excision. Plasmid DNA was linearised and transcribed *in vitro* by using T7 RNA polymerase. Xenopus oocytes were then injected

with 0.5 - 20 ng mRNA and subsequently assayed for deiodinase activity. Once a positive pool of clones was identified, a matrix approach was used to isolate a single clone encoding deiodinase activity. Using the outlined methods, these workers identified a 2.1 kilobase section of complementary DNA which coded for ID-I. The sequence of mRNA obtained from this cDNA sequence was shown to contain an in-frame uracil-guanine-adenine (UGA) codon at the 382-384 position. As describe above, the UGA codon is normally read as a stop codon, but can also serve as the codon for selenocysteine. Site directed mutagenesis of the UGA codon to UUA (code for leucine) or UAA (another stop codon) resulted in an inactive ID-I mutant protein being formed. Changing the UGA codon to UGU, which codes for cysteine resulted in a mutant ID-I with only 20% of the activity exhibited by the wild type enzyme (Berry *et al,* 1991b). As described previously a 3'ut sequence which allows the formation of a "stem loop" structure was found to be required for full translation of ID-I mRNA. Berry and coworkers also showed that two of the histidine residues (positions 158 & 174) in rat ID-I were important for maximum ID-I activity, with the histidine residue at position 174 found to be required for hormone binding (Berry, 1992). Subsequent cloning of the human ID-I has revealed that it also has a UGA codon at position 382, and the predicted amino acid sequence has 88% homology with the rat ID-I (Mandel *et al,* 1992).

ID-I was originally thought to occur only in the liver and kidney. However, ID-I activity has now also been found in a wide range of other tissues including skeletal muscle, heart, spleen, lung, intestine, lactating mammary glands, salivary glands, pituitary, brain and white adipose tissue. However, ID-I activity in these tissues is quite low, ranging between 0.1 and 5% of the activity found in liver and kidney. The thyroid also has an ID-I which appears to have even higher levels of activity than hepatic ID-I (Green, 1978; Erickson *et al,* 1981). Thyroidal ID-I has been shown to have similar reaction kinetics, sensitivity to PTU, substrate specificity, and mRNA identity to that of hepatic ID-I.

However, unlike hepatic ID-I whose expression can be induced by thyroid hormones, thyroidal ID-I expression is induced by TSH and thyroid stimulating immunoglobulins (TSIs) (Erickson *et al,* 1982; Wu, 1983; Ishii *et al,* 1981).

b) Type-II iodothyronine deiodinase (ID-II)

Unlike ID-I, ID-II can only catalyse 5'-deiodination, and hence lacks 5-deiodinase activity (table 1a). ID-II has been shown to be membrane bound, with a substrate binding subunit which exhibits a molecular weight of approximately 27 - 29 kDa. Gel filtration studies have suggested that native ID-II may have a molecular weight of approximately 200 kDa (Safran *et al,* 1991; Safran and Leonard, 1991). Studies into the substrate specificity of ID-II have revealed that T_4 is the preferred substrate, with rT_3 the other main substrate deiodinated by ID-II. As with ID-I, ID-II has an absolute requirement for reduced thiols (RSH). However, ID-II requires much higher concentrations of RSH, and as yet the physiological form of RSH has not been elucidated. The 5'-deiodination catalysed by ID-II is believed to follow "sequential type" reaction kinetics, although the exact mechanism is unknown. Sequential reaction kinetics imply that both the cofactor and substrate interact with ID-II prior to the formation of the product. ID-II has been shown to be insensitive to PTU inhibition at micromolar concentrations, and this confirms that ID-II does not utilise a hyperreactive Se^- group. However, radiocontrast agents do competitively inhibit ID-II as well as ID-I, although the mechanism of action is once again unclear.

The level of ID-II activity found in the brain, pituitary and brown adipose tissue (BAT) has been shown to decrease in selenium deficiency, and this observation originally led workers to believe that ID-II was a selenoenzyme (Beckett *et al,* 1989). It is noteworthy that when selenium and iodine deficiency occur together, brain ID-II activity increases, while pituitary and BAT ID-II activity decreases (Arthur *et al,* 1991a). The exact mechanism underlying this divergence of expression is as yet unknown, however levels of brain ID-II

have been shown to rise in thyroidectomized rats, and hence a change in thyroid hormone status is implicated (Chanoine *et al,* 1992). In contrast to Beckett and Arthur's observations, other workers using cultured glial cells have shown that ID-II activity is unaffected by the concentration of selenium in the growth medium (Safran *et al,* 1991). In addition, these workers could not demonstrate incorporation of ^{75}Se into ID-II when glial cells were grown in the presence of [^{75}Se]-selenite. Furthermore, ID-II has been shown to be 100-fold less sensitive to inhibition by gold thioglucose than ID (i.e. similar in sensitivity to the cysteine mutant of ID-I) (Berry and Larsen, 1992). Although ID-II has not been cloned as yet, the above evidence strongly suggests that ID-II is not a selenoenzyme. It is thought that the enzyme has a cysteine residue instead of selenocysteine at its active site. The decrease in ID-II observed by Beckett and Arthur in selenium deficient rats can be explained by elevation of plasma T_4 (Beckett *et al,* 1989). Selenium deficiency results in reduced ID-I activity and hence elevated plasma T_4. High plasma T_4 is believed to cause increased degradation of ID-II, resulting from increased inactivation and internalisation of the enzyme. T_4 promotes a rapid interaction of ID-II with F-actin stress fibres, and this results in the removal of ID-II and its transport to endosomes. In the absence of T_4, the internalisation of the enzyme is much slower and is directed to dense lysosomes by an F-actin independent pathway (Farwell and Leonard, 1989; Farwell *et al,* 1993).

The tissue distribution of ID-II is much more limited than ID-I, with only the brain, central nervous system (CNS), pituitary and BAT shown to express ID-II. The role of ID-II is though to be restricted to local production of T_3, though the T_3 status of these tissues has widespread effects on metabolism.

c) Type-III iodothyronine deiodinase (ID-III)

Little is known about ID-III. The enzyme is known to catalyse 5-deiodination in a reduced thiol-dependent manner utilising sequential reaction kinetics. Unlike the other IDs, ID-III appears to prefer T_3 to T_4 as the substrate for deiodination, and thus ID-III can be considered as an enzyme of thyroid hormones deactivation. ID-III is believed to be membrane bound, but its molecular weight has not been determined. The enzyme has been shown to be insensitive to PTU, but like the other IDs it is competitively inhibited by radiocontrast agents. Furthermore, like ID-II it is not thought to be a selenoenzyme. ID-III is found in the brain, CNS, placenta and skin, though its role is uncertain. In the placenta, ID-III may act to supply iodine to the foetus. Under normal conditions, ID-III is the main source of plasma rT_3 (Hennemann, 1986).

1.09 : Thyroidal ID-I and its role in thyroidal T_3 production

Under normal conditions the thyroid provides approximately 20% of plasma T_3, with the bulk of T_3 being derived from peripheral 5'-deiodination of plasma T_4 (Visser, 1988). The production of T_3 by the thyroid is generally thought to result from *de novo* synthesis (Adams and Larsen, 1973). However Green suggested that a system, independent of the thyroperoxidase (TPO) was responsible, at least in part for thyroidal T_3 production (Green, 1978). This system has been suggested to be ID-I, and the importance of ID-I in thyroidal T_3 production has now been reaffirmed (Laurberg, 1980; Laurberg, 1984). Laurberg using perfused dog thyroids showed that preferential accumulation of T_3 and rT_3 was inhibited to some degree by PTU, but not by methimazole. PTU and methimazole both inhibit thyroid hormone synthesis, but PTU in addition can inhibit ID-I. Hence, Laurberg argued that a significant proportion of T_3 was produced from 5'-deiodination of T_4.

In conditions which give rise to elevated plasma TSH such as iodine deficiency, there is a switch to thyroidal T_3 as the main source of plasma T_3 (Adams and Larsen,

1973). This switch is believed to be the main adaptive mechanism for limiting the serious effects of iodine deficiency (Silva, 1985). Iodine deficiency thus leads to a decrease in plasma T_4, while plasma T_3 is maintained. Presumably this decrease in plasma T_4 diminishes the negative feed back control of TSH secretion, exerted by T_4 at both the hypothalamus and the pituitary. Hence an increase in TSH secretion occurs, with a subsequent rise in plasma TSH. In addition to the observed increase in thyroidal T_3 production, the amount of T_4 produced is reduced. The mechanism by which plasma TSH causes these changes in thyroidal T_3 and T_4 production is under debate. The ratio of T_3 to T_4 on thyroglobulin has been shown to rise in iodine deficiency, and hence one proposed mechanism is that TSH mediates its changes via increased *de novo* synthesis of T_3 (Adams and Larsen, 1973). Presumably, as a result of lower thyroidal iodine concentration, the prevailing conditions favour the production of monoiodotyrosyl residues on thyroglobulin. Hence the probability of two diiodotyrosyl residues coupling to form T_4 is lowered, while the probability of a mono and a diiodotyrosyl residue coupling to form T_3 is increased.

Another hypothesis is that T_3 is increased through enhanced intrathyroidal 5'-deiodination of T_4 to T_3. The thyroid is known to contain ID-I, and TSH has been shown to stimulate the expression of ID-I via the cyclic-AMP second messenger cascade (Erickson *et al,* 1982; Wu, 1983; Ishii *et al,* 1983). Pazos-Moura and coworkers demonstrated in the rat that iodine deficiency can cause elevation of thyroidal ID-I activity (Pazos-Moura *et al,* 1991). As a result of their findings, these workers postulated that intrathyroidal 5'-deiodination of T_4 is an important mechanism in the preferential production of T_3 by the thyroid in iodine deficiency, a hypothesis also supported by other workers (Green, 1978; Laurberg, 1980; Laurberg, 1984; Chanoine *et al,* 1993).

In addition to 5'-deiodination, Laurberg also presented evidence for preferential secretion of T_3 (Laurberg, 1980). This effect was postulated to be due to more rapid

liberation of T_3 from thyroglobulin than T_4, however the effect was short lived and is therefore unlikely to contribute significantly in conditions which give rise to long term elevation of plasma TSH. A combination of increased *de novo* synthesis and thyroidal 5'-deiodination are therefore the most likely mechanisms which could give rise to the increased production of T_3 by the thyroid under TSH stimulation.

Various groups have studied the source of thyroidal T_3 using isolated human thyrocytes grown in monolayer or suspension culture. Ollis and coworkers demonstrated that T_3 could be secreted by thyrocytes in primary culture in response to stimulation by TSH (Ollis *et al,* 1985). However, this secretion of T_3 was found to result from the release of preformed T_3, with no evidence that human thyrocytes in culture could synthesis T_3. Other workers have proposed that T_3 secreted by thyrocytes can arise from *de novo* synthesis (Kraiem *et al,* 1988). Kraeim's findings were supported by Sato and coworkers who demonstrated iodine trapping, organification and release of thyroid hormones from follicular thyrocytes in suspension culture (Sato *et al,* 1988). Enhancement of these markers of thyroid function where observed in the presence of DMSO (1.7%) and low FCS (1%). These workers concluded that human thyrocytes can synthesis T_3 and T_4 *de novo* so long as they are not allowed to dedifferentiate. Hence they proposed that 1.7% DMSO and lower FCS (1%) prevented the dedifferentiation of the thyrocytes and thus allowed maintenance of normal function.

1.10 : Iodine, selenium and thyroid hormone status

In addition to selenium, iodine is also considered to be a trace element, found in group 7, row 5 of the periodic table. Iodine is required for thyroid hormone synthesis, and thus iodine deficiency has a profound effects on thyroid hormone status. The effects of iodine deficiency have been known for a long time, with iodine deficiency believed to cause two distinct forms of cretinism. Neurological cretinism is believed to be caused by maternal

iodine deficiency during the development of the fetus, and as its name suggests, involves severe impairment of mental development, though stunted growth is not usually observed. In contrast, myxoedematous cretinism is caused when iodine deficiency occurs during childhood. This form of cretinism results in stunted growth and some mental deficiency. Thus, both forms of cretinism result from hypothyroidism, but at different developmental stages, either as a fetus or as a child. Iodine deficiency causes plasma TSH to increase, plasma T_4 to decrease, while plasma T_3 tends to remain essentially unchanged. These changes are mediated by a series of events which are initiated by transient decreases in both plasma T_3 and T_4 resulting in decreased negative feed back of TSH secretion in the pituitary and elevated plasma TSH. Elevated plasma TSH causes an increase in thyroidal T_3 production, at the expense of T_4. Hence plasma T_3 levels are restored to near normal, while plasma T_4 decreases. The exact mechanisms which give rise to this switch to thyroidal T_3 production are not fully known, and are discussed above. Reduced plasma T_4 also has profound effects on the synthesis of growth hormone in the pituitary. Lower plasma T_4 in a child will result in a decreases in growth hormone synthesis, and hence the stunted growth observed in myxoedematous cretinism. Hypothyroidism during pregnancy can presumably decrease maternal plasma T_4 and so give rise to insufficient T_3 levels in the brain of the fetus, thus resulting in neurological cretinism (Delange, 1974; Hetzel *et al,* 1988).

In various regions of the world including Northwest China and Central Africa, both selenium and iodine deficiencies occur together and have been shown to have more profound effects on growth and development than iodine deficiency alone (Vanderpas *et al,* 1990; Goyens *et al,* 1987). Therefore selenium deficiency may prove to have an important role in the pathogenesis of myxoedematous endemic cretinism, by exacerbating some aspects of hypothyroidism associated with iodine deficiency (Arthur *et al,* 1990c). Indeed markers of hypothyroidism such as increased plasma TSH and decreased plasma

T_4 are exacerbated by concurrent selenium deficiency; changes which are most probably due to modification of the pituitary and hepatic deiodination pathways.

1.11 : Aims of the thesis

The aims of the thesis were to :

1) Optimise the conditions for the growth of human and sheep thyrocytes in primary culture, and subsequently determine the source of thyroid hormones secreted from these cultures.

2) Investigate species differences in thyroidal ID-I activity and expression.

3) Study the control of ID-I activity and expression by TSH and the activation of various second messenger systems using cultured human thyrocytes in primary culture.

4) Investigate the selenium-dependence of ID-I expression and activity in human thyrocytes grown in primary culture.

5) Determine the effects of selenium, iodine and combined selenium and iodine deficiencies on thyroidal ID-I expression *in vivo*.

Section 2 : MATERIALS AND GENERAL METHODS

2.01 : Equipment and Chemical Suppliers

Commercially available equipment and chemicals were obtained from the following sources:-

Amersham International plc, Northern Europe Region, Lincoln Place, Green End, Aylesbury, Buckinghamshire, U.K.

$Na^{125}I$ (specific activity, 44.4 MBq/μg); $Na_2(^{75}SeO_3)$ (specific activity, 74MBq/mg); L-3,3',5'-Tri-[^{125}I]-iodothyronine ([^{125}I]-rT_3) (specific activity, 44 MBq/μg).

Merck Ltd, Hunter Boulevard, Magna Park, Lutterworth, Leicestershire, U.K.

Acetic acid; acrylamide; ammonium solution; bromophenol blue; chloroform; dimethyl sulphoxide; dipotassium hydrogen orthophosphate; ethanol; glycerol; glycine; hydrochloric acid; hydrogen peroxide; 2-mercaptoethanol; methanol; N,N'-methylenebisacrylamide; microcrystalline cellulose; orthophosphoric acid; potassium iodide; potassium dihydrogen orthophosphate; sodium acetate trihydride; sodium borate; sodium chloride; sodium dodecyl sulphate; sulphuric acid; thin layer chromotography plates (cellulose-F, 5565); tris(hydroxymethyl) methylamine.

Bio-Rad Laboratories, Bio-Rad House, Maylands Avenue, Hemel Hempstead, Hertfordshire, U.K.

Low range molecular weight markers; N,N,N',N'-tetramethyl ethylenediamine (TEMED).

Boehringer Mannheim U.K., Lewes, Sussex, U.K.

Dispase (type II); ethylenediaminetetraacetic acid (EDTA).

ICN Flow, Unit 18, Thame Park Business Centre, Wenham Road, Thame, Oxfordshire, U.K.

Amphotericin B; bovine serum albumin (reagent grade, fraction V); Ca^{2+}/Mg^{2+} free Earle's balanced salt solution; Costar cell culture flasks (75 cm^3); Dulbeco's modified Eagle's medium (DMEM, w/o phenol red); Earle's balanced salt solution (EBS, w/o phenol red); 0.22 μm filters (sterile); L-glutamine; penicillin; streptomycin; 12 well plates.

Kodak Diagnostics Ltd, Mandeville House, 62 The Broadway, Amersham, Buckinghamshire, U.K.

L-[^{125}I]Thyroxine ([^{125}I]-T_4) (specific activity >44 MBq/μg); L-3,3',5-Tri-[^{125}I]-iodothyronine ([^{125}I]-T_3) (specific activity > 44 MBq/μg).

Lockertex, Locker Wire Weavers Ltd, P.O. Box 161, Church Street, Warrington, Cheshire, U.K.

Nylon gauze (100 and 30 μm).

Lorne Diagnostics, P.O. Box 6, Twyford, Reading, Berkshire, U.K.

Worthington Collagenase (type I)

National Institute for Biological Standards and Control, P.O Box 1193, Potters Bar, Hertfordshire, U.K.

Thyroid Stimulating Hormone (TSH) (NIBSC Code: 53/011).

Scottish Antibody Production Unit, Carluke, Lanarkshire, U.K.

Normal sheep serum; donkey anti-sheep serum; sheep anti-T_3 serum; sheep anti-T_4 serum.

Sigma Chemical Company Ltd, Fancy Road, Poole, Dorset, U.K.

Adenosine 5'-triphosphate; ammonium persulphate; 8-anilino-1-naphthalene sulphonic acid (ANS); 8-bromoadenosine 3'5'-cyclic monophosphate; bromoacetic acid n-hydroxy-succinimide ester; controlled process serum replacement-5 (CPSR-5); Coomassie brilliant blue (R-250 and G-250); cyanogen bromide; 5,5'-diethyl-barbituric acid (barbitone); dipotassium hydrogen orthophosphate; dithiothreitol; gelatin; gold thioglucose; glutathione (reduced form); glutathione reductase; iopanoic acid; methimazole; N,N-dimethylformamide; nicotinamide adenine dinucleotide phosphate (type X); phosphate buffered saline (PBS) powder; propylthiouracil; Sephadex G25; Sephadex LH20; sodium selenite; tert-butanol; trichloroacetic acid; triethylamine; trypsin (type III); x-ray film (Kodak X-OMAT XAR-5 film).

2.02 : Materials obtained from non-commercial sources

Normal human serum used to quench the ID-I assay was kindly supplied by Mr A. Jordan (Scottish Blood Transfusion Service). Human thyroid tissue was obtained with the help of Dr A. Toft, Dr K. MacLaren, Mr D. Lee, Sister R. Macmillan, the staff of Theatre 4 at the Royal Infirmary of Edinburgh and the staff of Edinburgh University Department of Pathology.

2.03 : Isolation and culture of thyrocytes

Human thyrocytes were isolated from human thyroid tissue (surplus to routine examination by a pathologist) from patients undergoing thyroid surgery, whilst ovine thyrocytes were isolated from sheep thyroids obtained from the local abattoir. Cells were isolated using a modified version of a dog thyroid cell culture method (Rapoport, 1975) as previously described (Beech *et al,* 1993). The tissue was finely minced with scissors and the resulting fragments were then washed four times with EBS before digestion for 2 h in 50

ml of an enzyme cocktail containing dispase (0.5% w/v), trypsin (0.25% w/v), collagenase (0.1% w/v) and BSA (2% w/v) in EBS. Following digestion, an equal volume of EBS was added and the mixture filtered through a 100 μm mesh gauze to remove undigested tissue. The resulting filtrate, containing released thyroid cells, was centrifuged at 125 g for 15 min to pellet the thyrocytes and leave the majority of erthyrocytes in suspension. The pellet was resuspended in EBS and re-centrifuged twice, and the cells finally resuspended in 50 ml of DMEM containing 10% (v/v) CPSR-5 (fetal calf serum treated to remove immunoglobulins and endotoxins), penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (2.5 μg/ml) and added glutamine (2 mmol/L). The cell suspension was then filtered through a 30 μm mesh gauze and the cell yield measured with a modified Neubauer haemocytometer.

Finally the thyrocytes were plated out in DMEM/10% CPSR-5 into 12-well plates (2.5 cm diameter) at a density of $5x10^5$ cells/well in 1 ml of medium, or into 75 cm^2 flasks in 20 ml of medium at a density of $10x10^6$ cells/flask. The cells were incubated at 37°C in an atmosphere of 5% CO_2 for 24 h before the medium was changed and any additions made.

2.04 : Radioimmunoassay of thyroid hormones

a) Preparation of radioimmunoassay buffer

The RIA buffer was prepared by dissolving 18.42g of barbitone in 2L of distilled water using gentle heat and agitation to obtain a 45 mmol/L barbitone buffer. To 100 ml of the barbitone buffer, 0.2g of thiomersal and 2.0g of gelatine were added and allowed to dissolve using gentle heat before being returned to the bulk of the buffer. On cooling to room temperature the pH of the buffer was adjusted to pH 8.6 using 10M NaOH and the buffer stored at 4°C.

b) Preparation of pre-precipitated radioimmunoassay antibodies

Pre-precipitated anti-T_3 and anti-T_4 antibodies were prepared by adding 0.5 ml of the appropriate sheep serum antibody to 0.5 ml of normal sheep serum and 15 ml of donkey anti-sheep serum. The mixture was incubated for 1 hour at room temperature before being gently centrifuged (180g) for 5 mins, to pellet the pre-precipitated antibody. The pre-precipitate was washed twice with barbitone buffer and stored at 4°C for use.

To determine the titre of antibody required for a sensitive radioimmunoassay an antibody dilution curve was performed. A series of 1:2 dilutions of the antibody were performed and 200μl of each of the dilutions were added to 600μl of tracer (see Section 2.04c). Following a 2 hour incubation at room temperature the samples were centrifuged at 2400g for 30 mins. The supernatant was then tipped off and the tops of the tubes blotted dry before the tubes were counted for ^{125}I activity (one tube was left untipped as a blank). The dilution factor was then plotted against the % of counts retained (compared to the blank) and the dilution factor which would yield 60% binding was determined (example, figure 2a).

Non-specific binding (NSB) reagent was prepared in a similar way as the anti-thyroid hormone pre-precipitated antibodies, but using 1 ml of normal sheep serum and 15 ml of donkey anti-sheep serum only. The NSB was then diluted 1:40 and used to dilute the pre-precipitated antibodies to give a working stock solution, with binding of approximately 60% in the absence of antigen.

c) Radioimmunoassay of thyroid hormones

The pre-precipitate RIA assays used to determine T_3 and T_4 are those previously described (Beckett *et al,* 1987), and are summarised below: Thyroxine and triiodothyronine stock solutions (1 mmol/L T_4 and T_3 respectively) were prepared in methanol and a range of standards prepared (T_4, 0 - 250 nmol/L; T_3, 0 - 20 nmol/L) by dilution of the stock solutions

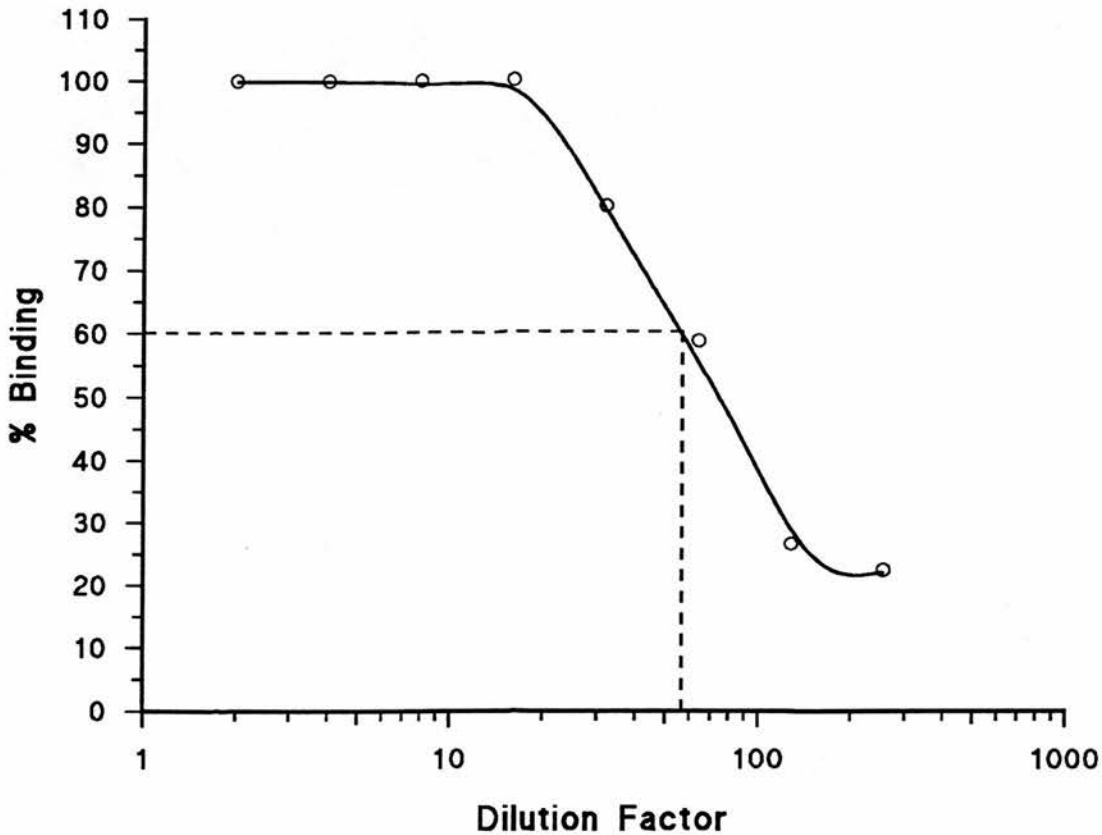


Figure 2a : An example of an antibody dilution curve used to determine the dilution factor required to give a sensitive RIA for T_3 and T_4.

with EBS. These standards were used to construct a standard curve each time the assays were performed.

Working radiolabelled tracer was prepared by adding 74 kBq of the appropriate radiolabel to 100 ml of barbitone buffer containing 0.01% (w/v) ANS. Sample or standard (50μl) was added to 600μl of working tracer, before pre-precipitated antibody (200μl) was added and the tubes agitated. Following a 3h incubation the tubes were centrifuged for 30 min (4°C, 2400g), the supernatant decanted, and the pellet counted in a Nuclear Enterprises, NE1600 gamma counter. Results were then interpolated by the SASPro Immunoassay D.P. program of P.R. Edwards, Department of Molecular Endocrinology, Middlesex Hospital, London, U.K.

d) Production of solid-phased T_3/T_4 antibodies

Sufficient water was added to 20g of microcrystalline cellulose to produce a slurry light enough to be kept well mixed by a magnetic stirrer and "flea". The pH was adjusted to between 10 and 11, and maintained within this range using 1M NaOH as 1g of cyanogen bromide was slowly added. After approximately 10 min, the slurry was poured onto a glass sinter and washed 3 times with 500 ml of a 0.1M $NaHCO_3$ solution. The slurry was washed once with 500 ml of borate buffer (4.763g of sodium borate with 135 ml of 0.1M HCl, made up to 1 litre, pH 8.6), before being made into a slurry once again in borate buffer. Reconstituted antibody in borate buffer was added to the slurry at a ratio of 1 ml of antibody, to 2g of the original dry cellulose and the mixture incubated overnight with gentle agitation. The antibody/cellulose slurry was centrifuged (300g, 10 min) and the pellet washed with 50 ml of acetate buffer (4.9g sodium acetate trihydrate with 9.4 ml glacial acetic acid in 1 litre of water, pH 4). Following centrifugation, the pellet was washed with 50 ml glycine buffer (0.1M, with 2 g/L gelatine, 100 mg/L thiomersal, pH'd to 10.5 with NaOH) and centrifuged once again. This protocol of alternate washing with

acetate and glycine buffers was repeated twice more, with the slurry remaining in glycine buffer at the end of the wash protocol. The slurry was then diluted 1:100 with glycine buffer on the basis of the volume of antibody used (e.g. 5 ml antibody, 500 ml final volume). An antibody dilution curve was then constructed for each solid-phased antibody as described in Section 2.04b to determine the dilution factor required for a % binding of 60%.

2.05 : Assay of ID-I activity in homogenates

a) [^{125}I]-rT_3 method

Tracer was purified before use to reduce contamination by free [^{125}I]-iodide. This reduced the background to the assay and produced a more sensitive assay. Purification of the tracer was performed by passing the [^{125}I]-rT_3 tracer through a small (approx 2 ml) Sephadex G25 column equilibrated with 100 mmol/L potassium phosphate buffer (pH 7.4) containing 1 mmol/L EDTA. [^{125}I]-iodide was eluted by approximately 10 ml of the assay buffer. The [^{125}I]-rT_3 was then eluted using a 1% (w/v) BSA solution in assay buffer, with the recovery of [^{125}I]-rT_3 between 50% and 85% depending on the age of the tracer. The purified tracer was stored at 4°C for upto a week before use.

ID-I activity was determined by measuring the rate of ^{125}I released from [^{125}I]-rT_3 (Sawada *et al,* 1986). The activity of ID-I in tissue samples was determined as follows: The tissue was homogenized in assay buffer to obtain a 20% (w/v) homogenate. After centrifugation in a Beckman GS-6R benchtop centrifuge for 15 min at 300g, the supernatant was removed and the ID-I activity assayed by incubating 200μl of supernatant sample with 100μl of purified tracer (3.7kBq [^{125}I]-rT_3 with non-isotopic rT_3 added to a final rT_3 concentration of 2 nmol/L) in the presence of 5 mmol/L DTT. Following a 15 min incubation the reaction was stopped using 0.5 ml of human serum (which binds the rT_3 and T_2) and the protein in the sample precipitated by adding 0.5 ml 10% (w/v)

trichloroacetic acid (TCA). The precipitated protein, together with the bound tracer, were then pelleted by centrifugation (1500g, 10 min) and 0.5 ml of the supernatant (s) along with the remaining supernatant and pellet (p) were counted for ^{125}I activity using an Nuclear Enterprises, NE1600 gamma counter. The % of ^{125}I released from [^{125}I]-rT_3 was determined using the following formulae (s÷(s+p)x100) and corrected for the blank (buffer and tracer only). If the % release of ^{125}I exceeded 20% which is the effect range of the assay, the sample was diluted so that the % change stayed between 0.2 and 20. The amount of ^{125}I released was calculated using the following correction factor; 1% released = 4.6 fmols rT_3 deiodinated.

b) Non-isotopic T_4 method

The tissue was homogenised in assay buffer as described in Section 2.05a to produce a 20% homogenate. To 450μl of homogenate, 50μl of T_4 (0.5 mg/ml) was added to start the assay. The reaction was stopped at time zero, and 10 min using 1 ml of ethanol. Subsequently, the precipitated proteins were pelleted by centrifugation at 2400g for 15 min. The supernatant was then removed and diluted 1:10 with barbitone buffer and assayed for T_3 using the RIA described in Section 2.04c. The amount of T_3 in the time zero sample was then subtracted from the 10 min sample to yield the amount of T_3 produced in 10 min. Subsequently the activity of ID-I in the sonicated sample was calculated.

2.06 : Assay of ID-I activities in cell sonicates

At the end of the incubation time, the medium bathing the thyrocytes was removed and the cells washed twice with 1 ml of EBS. The cells were then removed from the plate in 1 ml of ID-I assay buffer using a rubber policeman. Each of the cell suspensions were sonicated for approximately 5 seconds before the ID-I activity was determined by the [^{125}I]-

rT_3 method using 200μl of cell sonicate under the same conditions as described in Section 2.05a. The incubation time was altered where necessary to allow a significant level of ^{125}I production.

2.07 : Protein determination

All protein determinations were carried out using the Bradford dye-binding method (Bradford, 1976), adapted for use on a Cobas Fara (Roche Diagnostics, Welwyn Garden City, U.K.) centrifugal analyzer. Coomassie brilliant blue (G250) was prepared as described by Bradford except that the reagent was filtered before use through a Whatman grade 1 filter paper. Reagent (256μl) was added to each cuvette and incubated at 37°C for 100 s, with an initial absorbance reading (595 nm) taken at 95 s. After the addition of the sample (25μl) and water diluent (50μl), the cuvettes were incubated at 37°C for a further 180 s, prior to a final absorbance reading taken at 595 nm.

A standard curve was also constructed using bovine serum albumin at concentrations from 0 to 100 mg/L. The change in absorbance due to the standard was calculated and then used to plot a standard curve. The protein concentration of unknown samples were then interpolated from this curve. Samples which had protein concentrations above 100 mg/L were diluted with distilled water to allow the absorbance change to lie within the range of the standard curve.

2.08 : Preparation and use of N-Bromoacetyl-[^{125}I]-rT_3 affinity label

The N-Bromoacetyl-[^{125}I]-rT_3 affinity label was synthesized using a modified version (Arthur *et al,* 1990a) of the Nikodem method (Nikodem *et al,* 1980). 200μl of [^{125}I]-rT_3 (specific activity > 44 MBq/μg rT3) was added to an Ependorf tube and evaporated to dryness under a stream of dry nitrogen. To this, 20μl of a 1.5 mg/ml solution of bromoacetic acid n-hydroxy-succinimide ester in dimethylformamide was added, followed by 5μl of 10%

triethylamine in dimethylformamide. Following a 50 min incubation the mixture was purified by applying it to a small (approximately 2 ml) Sephadex LH20 column previously equilibrated with 0.1 mol/L HCl. Unreacted [^{125}I]-rT_3 was eluted first using 0.1 mol/L HCl and finally the affinity label was eluted using 95% ethanol. The affinity label wasstoredat -20°C in a sealed vial prior to use for upto 2 months. (N.B. The affinity label and all the materials used for its synthesis must be kept free of moisture, to avoid corruption of the synthesis).

Samples were diluted with 50 mmol/L Tris/HCl, 3 mmol/L EDTA, 3 mmol/L DTT buffer before 50μg of protein in a volume of 50μl was added to a tube containing 7.4kBq of dried label (dried under dry N_2). The label was allowed to react for 15 min before being diluted 1:3 with "boiling mix" consisting of SDS (35 mmol/L), glycerol (1.4 mmol/L), 2-mercaptoethanol (0.3 mmol/L) and bromophenol blue (15 mmol/L) and the samples heated to 90°C for 10 min. The samples were then loaded on an sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (SDS/PAGE) gel and the proteins separated and then visualised using autoradiography.

2.09 : SDS/Polyacrylamide-gel electrophoresis

SDS/PAGE was carried out in gels (0.1 cm x 16.5 cm x 18 cm) at room temperature using a Protean II electrophoresis system (Bio-Rad Laboratories Ltd, Watford, Hert, U.K.) in the presence of 1% SDS (w/v), using the buffer system described previously (Laemmli, 1970). The "resolving gel" was 14 cm long and consisted of 12% (w/v) polyacrylamide and 2.6% (w/v) NN'-methylenebisacrylamide in 375 mmol/L Tris/HCl buffer (pH 8.8). The "stacking gel" was 2.5 cm long and comprised of 3% (w/v) polyacrylamide in a 125 mmol/L Tris/HCl buffer pH 6.8 and was formed on top of the "resolving gel" with a 15 well "comb".

Protein samples were prepared for electrophoresis by heating at 90°C for 10 min in "boiling mix". The desired volume of the "boiling mix" was applied to each well in the

"stacking gel" and electrophoresis carried out at 50 mA, through the "stacking gel" and then at 25 mA, through the "resolving gel". The current was stopped when the bromophenol blue marker was within 0.5 cm of the end of the gel. The gel was stained with a 0.2% (w/v) Coomassie Brilliant Blue R in a water/methanol/acetic acid (50:50:7, by vol.) solution and destained in several changes of a water/methanol/acetic acid (88:5:7 by vol.) solution for 2 days.

The stained gel was then "sandwiched" between 2 sheets of gel drying film and dried using a Genevac CVP50 vacuum pump (Genevac, England, U.K.) in conjunction with a Rapidry, programable, heated gel drier (Atto, U.K.). The molecular weights of the standard proteins were plotted against the distance travelled and a standard curve drawn (e.g. figure 2b); from this curve, the molecular weights of the unknown proteins were determined (ID-I shown as an example).

2.10 : Autoradiography of SDS/PAGE gels

Autoradiography of SDS/PAGE gels was performed using Kodak X-OMAT XAR-5 X-ray film, and consisted of placing the dried gels in close contact with the film in an exposure cassette. Exposure of the film to the gels at -70°C was varied so that a sharp image was produced (15h - 4 days). All work with the undeveloped film was performed under an Ilford 914 filtered safety light (7.5 watt). Exposed x-ray films were kindly processed by the staff of the Department of Radiology in the Royal Infirmary of Edinburgh.

2.11 : Statistical analysis of data

Unless stated otherwise, all data in this thesis was subjected to a unpaired Student t-test, using the Instat statistical computer software package manufactured by Graph Pad Software, San Diego, CA, USA. Where a statistically significant difference occurred, the p value describing the event is denoted by * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$).

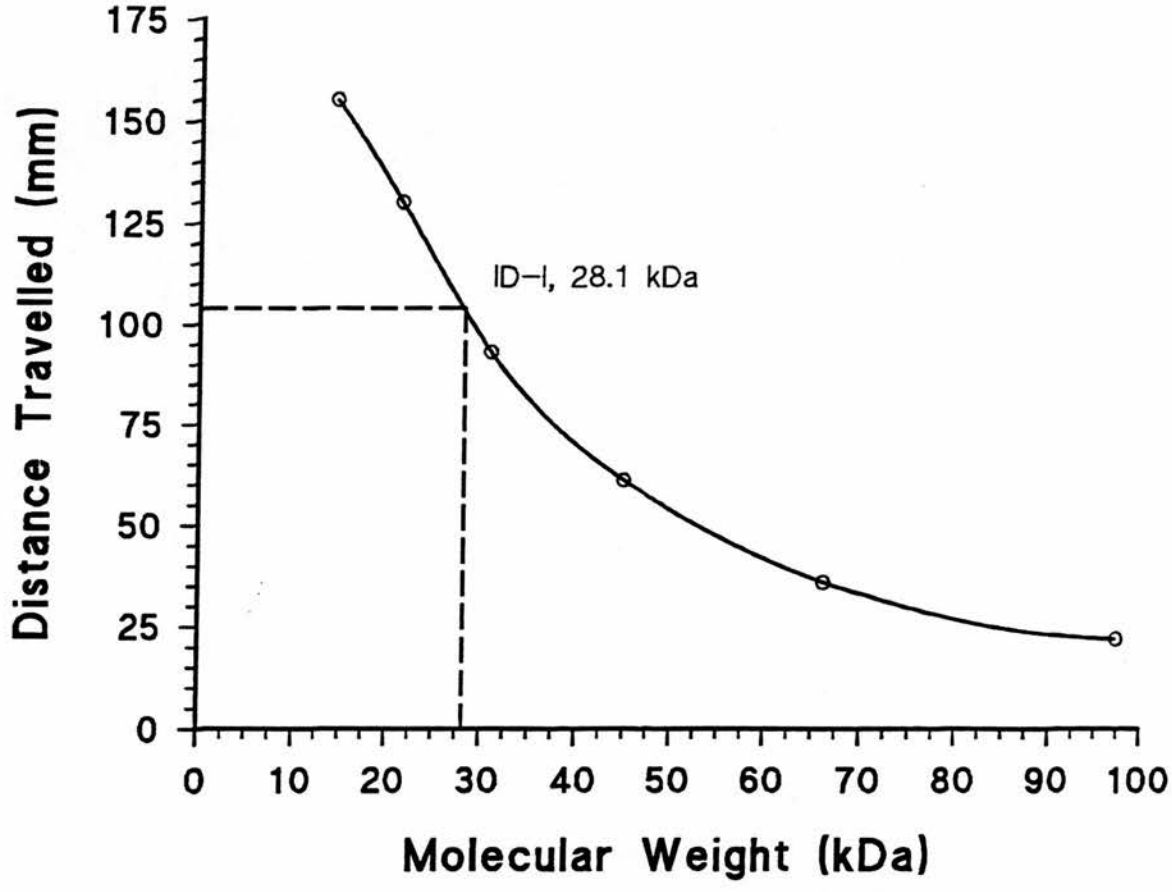


Figure 2b : An example of a molecular weight vs distance travelled plot used to determine the molecular weight of unknown bands on a 12% SDS-PAGE gel.

Section 3 : PRODUCTION OF THYROID HORMONES BY SHEEP AND HUMAN THYROCYTES IN PRIMARY CULTURE

Under normal circumstances, approximately 80% of plasma T_3 is produced from plasma T_4 as a result of 5'-deiodination in peripheral tissues (e.g. liver and kidney). These tissues have been shown to contain type-I 5'-iodothyronine deiodinase (ID-I) which is responsible for this 5'-deiodination and is known to be a selenoenzyme, containing stoichiometric amounts of selenium (Beckett *et al,* 1987; Behne *et al,* 1990; Berry *et al,* 1991b; Safran *et al,* 1990). The remaining 20% of plasma T_3 arises directly from the thyroid. However, in conditions where plasma thyrotropin (TSH) is elevated, such as in iodine deficiency, the thyroid becomes the major source of plasma T_3 (Adams and Larsen, 1973). It is unclear wether this increase in thyroidal T_3 production arises from the release of T_3 from thyroglobulin, synthesized in a *de novo* fashion (Visser, 1988) or from the action of thyroidal ID-I on thyroidal T_4 (Laurberg, 1984).

In primary culture, human thyrocytes produce T_3, and it has been suggested that the T_3 secreted by cultured human thyrocytes arises from *de novo* synthesis (Kraiem *et al,* 1988), particularly if a follicular preparation is used and dimethylsulphoxide (DMSO) and lower than usual concentrations of fetal calf serum are present in the culture medium (Sato *et al,* 1988). Other workers have suggested that the T_3 arises from release of preformed intracellular T_3 (Ollis *et al,* 1985). In contrast to human cultured thyrocytes, sheep thyrocytes appear to produce both T_3 and T_4 (Becks *et al,* 1987).

In this section I have studied the effects of TSH, KI and various inhibitors of ID-I and *de novo* synthesis on T_3 production by human and sheep thyrocytes grown in primary culture in order to investigate the significance of thyroidal deiodination of T_4 as a possible source of T_3 production in this system. *De novo* synthesis of T_3 and T_4 was also

investigated by studying the incorporation of ^{125}I into T_3 and T_4 using thin layer chromatography (TLC) and autoradiography.

3.01 : Methods

a) Time-course of T_3 and T_4 production by sheep thyrocytes

Sheep thyrocytes were prepared as described in Section 2.03. After 24 h, the medium was removed from the thyrocytes in monolayer culture and the cells washed twice with 1 ml of EBS before 1 ml of fresh DMEM/10% CPSR-5 growth medium was added. Various combinations of potassium iodide (KI, 1 μM) and TSH (1 U/L) were added to the thyrocytes, so that 4 groups were created, a) Control (KI -, TSH -); b) KI +, TSH -; c) KI -,TSH +; and d) KI + TSH + (+/- denotes presence/absence of TSH or KI). The thyrocytes were then incubated at 37°C in a 5% CO_2 incubator, and the growth medium from triplicate wells removed after 2, 4, 6 and 8 days of incubation. The concentration of T_3 and T_4 in the culture medium for each time point was determined by specific in-house radioimmunoassays (Section 2.04c).

b) Effect of TSH and KI on T_3 and T_4 production by human and sheep thyrocytes

Human and sheep thyrocytes were prepared as described in Section 2.03. After 24 h, the medium was removed from the thyrocytes in monolayer culture and the cells washed twice with 1 ml of EBS to remove unattached/non-viable thyrocytes and cytoplasmic contaminants from damaged thyrocytes. Fresh DMEM/10% CPSR-5 growth medium (1 ml/well) was then added to the thyrocytes. To wells in triplicate a range of TSH doses (0 - 10 U/L) were added in the presence of a fixed concentration of KI (10 μM).

In other experiments, thyrocytes were incubated in the presence of a range of KI doses (0 - 100 μM) with a fixed concentration of TSH (1 U/L) present. Following an

incubation period of 5 days the concentrations of T_3 and T_4 secreted into the culture medium were determined by the respective in-house radioimmunoassays (Section 2.04c).

c) Effect of IPA, PTU and methimazole on T_3 and T_4 production by human and sheep thyrocytes

Human and sheep thyrocytes were washed and provided with fresh growth medium as described in Section 3.01b, but with the addition of both TSH (1 U/L) and KI (10 μmol/l) to the medium (optimum concentrations for TSH and KI were determined from the above experiments). Increasing doses of a range of different inhibitors were then added to wells in triplicate. The inhibitors used were iopanoic acid (IPA, an ID-I inhibitor), methimazole (an inhibitor of T_3 and T_4 *de novo* synthesis) or 6-n-propyl-2-thiouracil (PTU, an inhibitor of ID-I activity as well as *de novo* synthesis). The thyroid hormone concentration in the overlying medium after a further 5 days was determined using specific radioimmunoassays for both T_3 and T_4 as previously described (Section 2.04c), and the results expressed as the amount of T_3 or T_4 produced per well per 5 days. To confirm that there were no significant changes in protein resulting from the various additions, the previously described additions were also made to other human and sheep thyrocytes. After a further 5 days in culture, the thyrocytes were washed twice and the removed from the wells in 1 ml of ID-I assay buffer using a rubber policeman. Subsequently, the thyrocytes were sonicated and the protein content of the sonicates determined as described in Section 2.07.

d) Effect of dimethylsulphoxide and fetal calf serum on T_3 and T_4 production from human thyrocytes in primary culture.

Human and sheep thyrocytes were prepared, cultured for 24 h and washed as described in Section 3.01a. Fresh medium was added back to the washed thyrocytes, with various

additions, made such that 4 groups were created : i) 10% CPSR-5, DMSO -; ii) 10% CPSR-5, DMSO +; iii) 1% CPSR-5, DMSO -; and iv) 1% CPSR-5, DMSO + (all thyrocytes were grown in the presence of 1 U/L TSH and 10μM KI, DMSO +/- denotes the presence/absence of 1.7% DMSO). Following an incubation period of 5 days, the concentrations of T_3 and T_4 secreted in the culture medium were determined by the respective in-house radioimmunoassays (Section 2.04c).

e) Incorporation of ^{125}I into thyroid hormones by human and sheep thyrocytes

Human and sheep thyrocytes were isolated as described in Section 2.03 and plated out in 75cm^2 flasks in 20 ml DMEM/10% CPSR-5 at a density of 10 million cells per flask. After 24 h the medium was removed, the cells washed twice with 20 ml EBS before fresh medium (DMEM/10% CPSR-5) was added containing 1.85 MBq of Na^{125}I (specific activity $\geq$ 44.4 MBq/μg) with or without TSH (1 U/L). After a further incubation for 5 days, the medium was removed and the T_3 and T_4 was immunoprecipitated from the medium by incubation with excess solid-phased anti-T_3/T_4 immunoglobulins for 15 h at 25°C (solid-phased immunoglobulins produced as described in Section 2.04d). The samples were then centrifuged at 180 g for 15 min, and the antibody pellet washed with 20 ml KI (1M) and re-centrifuged. The pellet was then resuspended in 5 ml of methanol and the suspension left for 15 h at 25°C to extract the bound hormones. After a further centrifugation at 180 g for 15 min, the methanolic supernatant was evaporated to dryness and the residue, containing immunoextracted T_3 and T_4 taken up in 100μl methanol, with 40μl used per sample.

The 40μl samples were then loaded onto a 20 cm square cellulose TLC plate and run for approximately 20 h using chloroform : tertiary butanol : ammonia (2M), (60:376:70 by vol.) as the developing solvent. Appropriate ^{125}I standards of T_3 and T_4 were also run on the same plate. No detectable Na^{125}I was carried over onto the TLC plates, as

determined by autoradiography. Once the TLC plate had dried, autoradiography was employed to demonstrate the presence of iodinated T_3 and T_4 as described for dried SDS/PAGE gels (Section 2.10).

3.02 : Results

a) Time-course of T_3 and T_4 production by sheep thyrocytes

In the absence of TSH and KI, the amount of T_3 and T_4 produced by the sheep thyrocytes was low, and slowly increased with the length of the incubation (figure 3a). Addition of KI alone had little effect, though a very slight increase was seen in both thyroid hormones above that observed in the control. The addition of TSH alone caused increases in both T_3 and T_4, with maximum T_3 and T_4 seen after 8 days of incubation. A similar pattern was seen when KI and TSH were added together, though the amount of T_3 and T_4 produced increased by more than 10-fold compared to the TSH + KI - triplicate after 8 days incubation.

b) Effect of TSH and KI concentration on T_3 and T_4 production by human and sheep thyrocytes

T_3 and T_4 production by sheep thyrocytes showed a clear dose-dependence on TSH, with the observed maximum production seen at a TSH concentration of 1 U/L (figure 3b). Human thyrocytes also produced T_3 in a dose dependent manner with respect to TSH, with maximum T_3 production observed at 1 U/L TSH (figure 3c). However, T_4 production by human thyrocytes was low and failed to be stimulated by TSH. In fact the T_4 concentration in the medium decreased when TSH was present at 1 U/L (figure 3c).

The production by sheep thyrocytes of both thyroid hormones increased with the concentration of KI, with the maximum production of both hormones observed at 10 μM (figure 3d). Concentrations of KI > 20 μM caused significant decreases in T_3 and T_4

production when compared to that observed at 10 μM (figure 3d). The production of T_3 and T_4 by human thyrocytes was unaffected by KI, with no significant change in the concentration of either thyroid hormone found (figure 3e).

c) Effect of IPA, PTU and methimazole on T_3 and T_4 production from human and sheep thyrocytes

In sheep thyrocytes, methimazole and PTU produced a dose-dependent inhibition of both T_3 and T_4 accumulation, with a maximal effect seen for both compounds at 1 mM (figure 3f & 3g). At this dose, methimazole caused a 35% decreases in T_3 and T_4, while PTU caused a 50% decreases in both T_3 and T_4. IPA had no significant effect on T_3 and T_4 accumulation in the growth medium bathing the thyrocytes (figure 3h).

In human thyrocytes, methimazole caused on significant change to the accumulation of T_3 in the medium, while the amount of T_4 decreased slightly. In contrast to the sheep thyrocytes, PTU and IPA caused a significant increase in T_4 and a corresponding decrease in the accumulation of T_3 (figure 3j & 3k). Maximal effects occurred at 10 mmol/L PTU which caused a 50% increase in T_4 and an 80% decrease in T_3 (figure 3j). In the case of IPA, maximal effects occurred at 100 μmol/L IPA and consisted of an 80% increase in T_4 and a 70% decrease in T_3 (figure 3k).

No significant change in the protein content of the wells could be seen when sheep and human thyrocytes were exposed to the various additions (human thyrocytes shown, figure 3l).

d) Effect of dimethylsulphoxide and fetal calf serum on T_3 and T_4 production by human thyrocytes in primary culture.

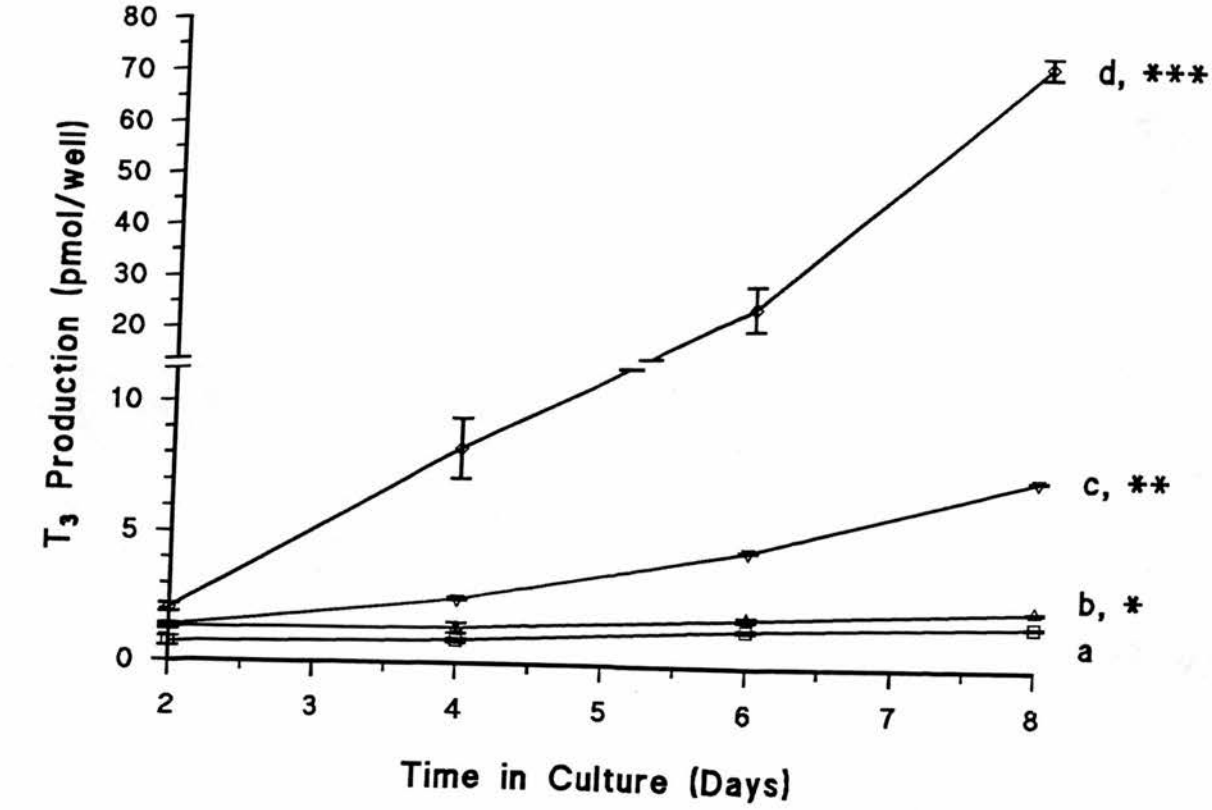
The addition of DMSO to the human thyrocytes produced no significant change in the amounts of both T_3 and T_4 secreted into the overlying medium, regardless of the

concentration of CPSR-5 (figure 3m). In comparison, the thyrocytes grown in the presence of 10% CPSR-5 exhibited higher levels of T_3 and T_4 into the overlying medium than those grown in the presence of 1% CPSR-5.

e) Incorporation of ^{125}I into T_3 and T_4 by human and sheep thyrocytes

In the presence of TSH, incorporation of ^{125}I into T_3 and T_4 occurred in TSH stimulated sheep thyrocytes, with no detectable incorporation occurring when TSH was absent (figure 3n). With human thyrocytes, no ^{125}I incorporation into T_3 and T_4 could be detected irrespective of the presence or absence of TSH.

Figure 3a : Time-dependent thyroid hormone production by sheep thyrocytes in primary culture with/without KI (10μM) and/or TSH (1 U/L) present. (a: TSH -, KI -; b: TSH -, KI +; c: TSH +, KI -; d: TSH +, KI +). The accumulated medium content of T_3 (□) and T_4 (Δ) was measured and the data expressed as mean ± SEM of triplicate wells for 2, 4, 6 and 8 days in culture (representive experiment, performed twice). Significant differences were observed across the time course and between the various additions on day 8 (indicated on figure).


80
70
60
50
40
30
20
10
5
0
T_3 Production (pmol/well)
d, ***
c, **
b, *
a
2
3
4
5
6
7
8
Time in Culture (Days)

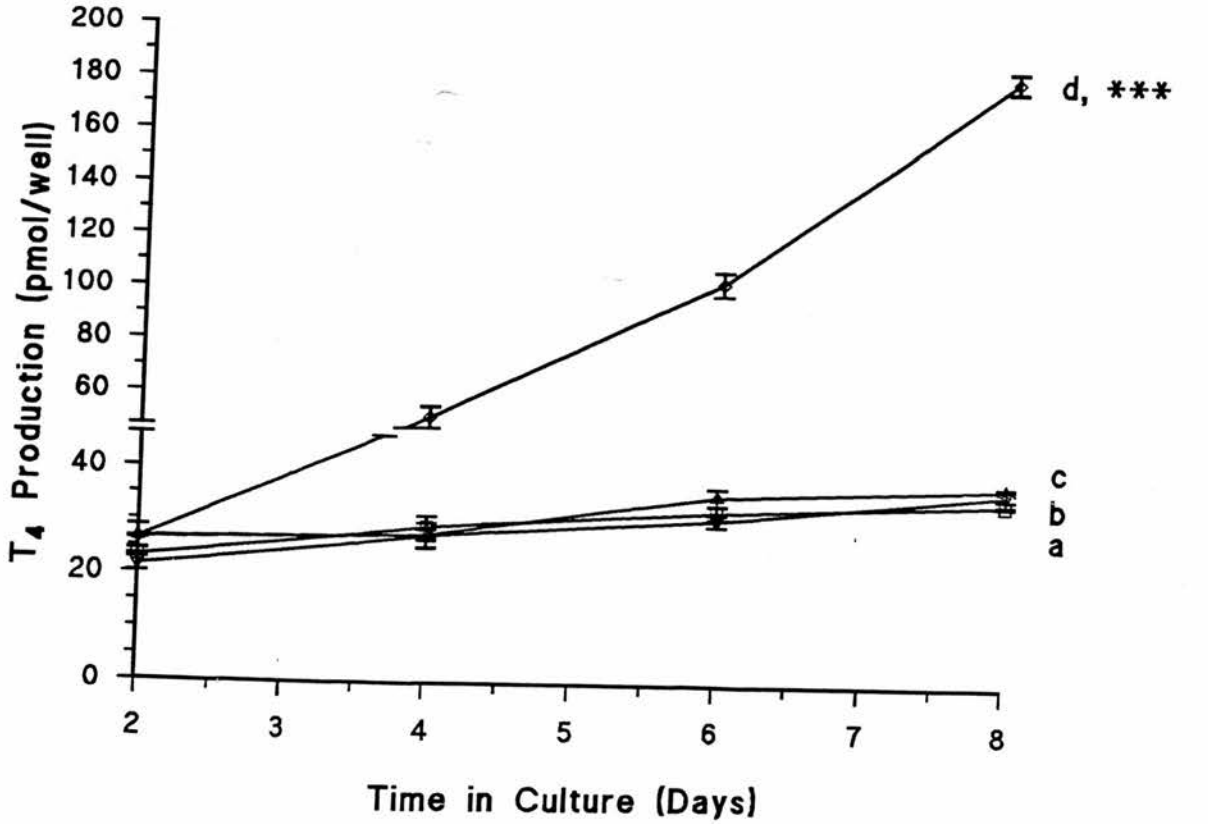
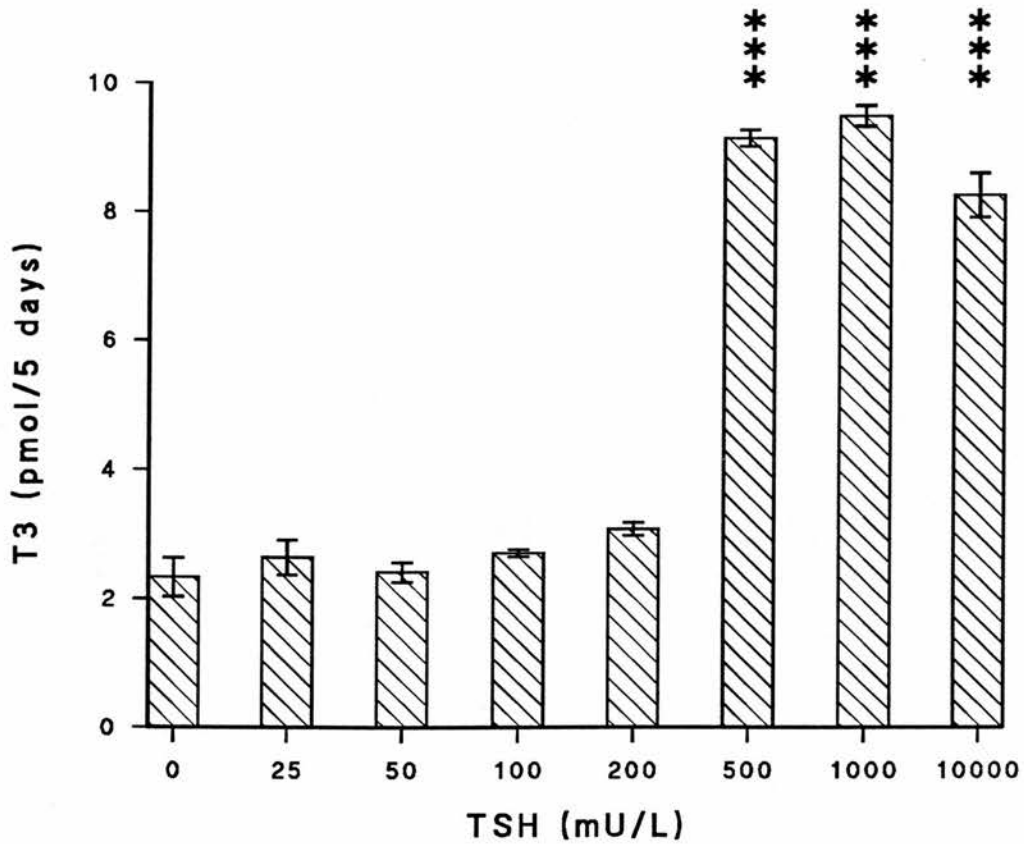

200
180
160
140
120
100
80
60
40
20
0
T_4 Production (pmol/well)
d, ***
c
b
a
2
3
4
5
6
7
8
Time in Culture (Days)

Figure 3b : Effect of increasing doses of TSH on thyroid hormone production from sheep thyrocytes in primary culture for 5 days in the presence of 10 μM KI. The accumulated medium content of T_3 and T_4 was measured and the data expressed as mean ± SEM of triplicate wells in a representative experiment carried out on at least three occasions. The accumulation of T_3 and T_4 with TSH concentrations above 500 mU/L was significant compared to the control (- TSH). A significant decrease was also seen between 1 U/L and 10 U/L ($p < 0.05$).


T3 (pmol/5 days)
0
2
4
6
8
10
0
25
50
100
200
500
1000
10000
TSH (mU/L)
****
****
****

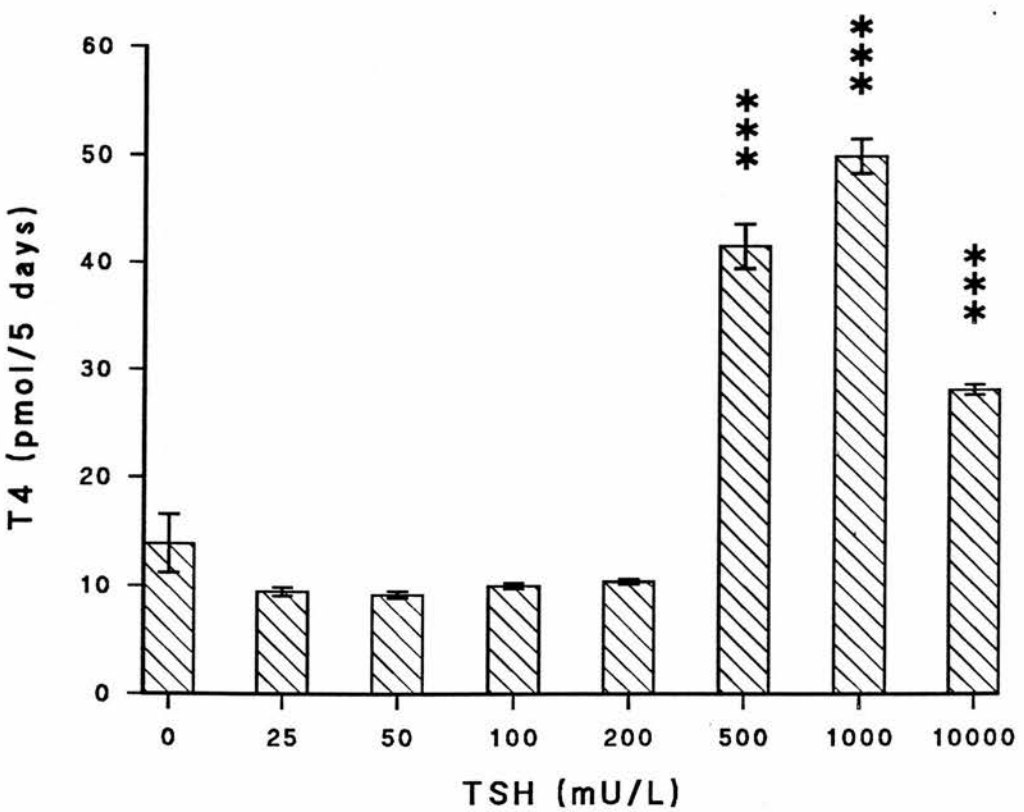
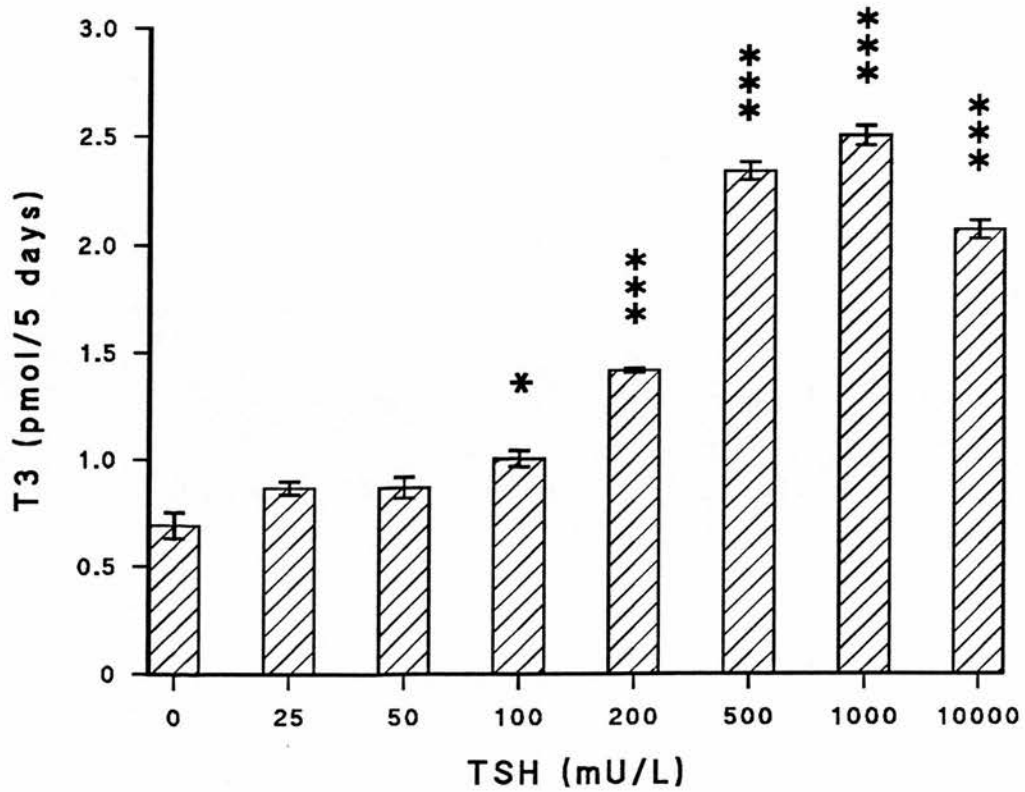

T4 (pmol/5 days)
0
10
20
30
40
50
60
0
25
50
100
200
500
1000
10000
TSH (mU/L)
****
****
****

Figure 3c : Effect of increasing doses of TSH on thyroid hormone production from human thyrocytes in primary culture for 5 days in the presence of 10 μM KI. The accumulated medium content of T_3 and T_4 was measured and the data expressed as mean ± SEM of triplicate wells in a representative experiment carried out on at least three occasions. The changes in accumulated T_3 and T_4 as TSH concentrations exceeded 500 mU/L were significant when compared to the control (- TSH). Significant changes were also seen between TSH concentrations of 1 U/L and 10 U/L ($p < 0.05$).


T3 (pmol/5 days)
3.0
2.5
2.0
1.5
1.0
0.5
0
*
***
***
***
***
0
25
50
100
200
500
1000
10000
TSH (mU/L)

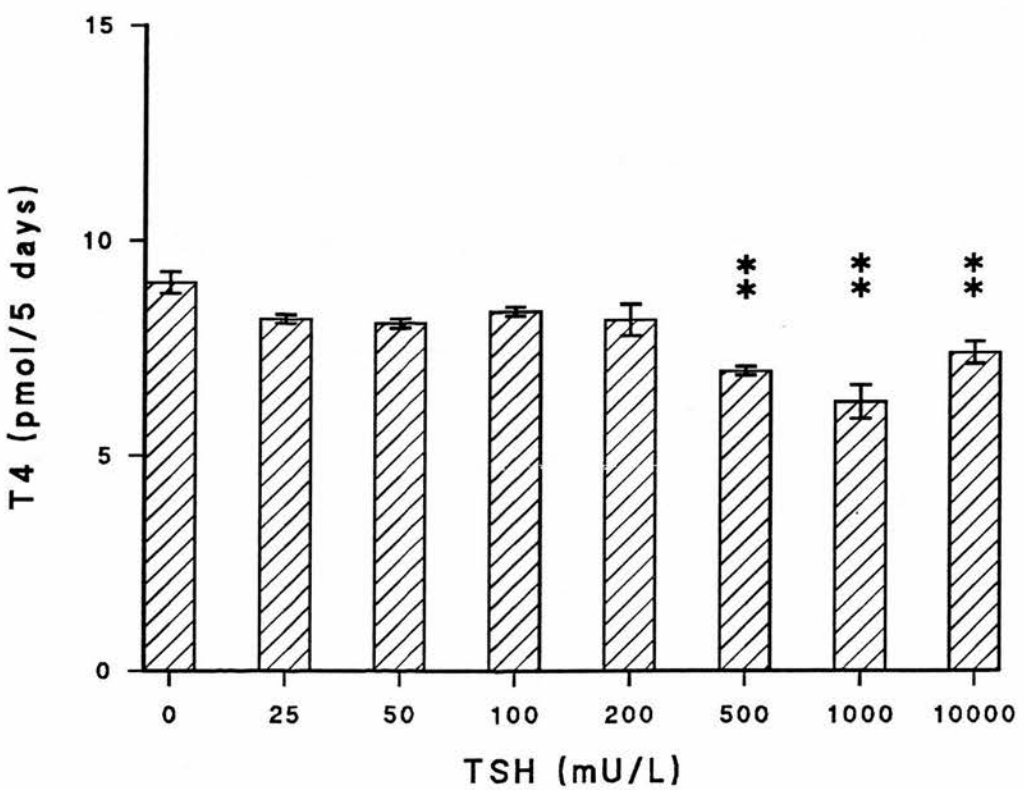
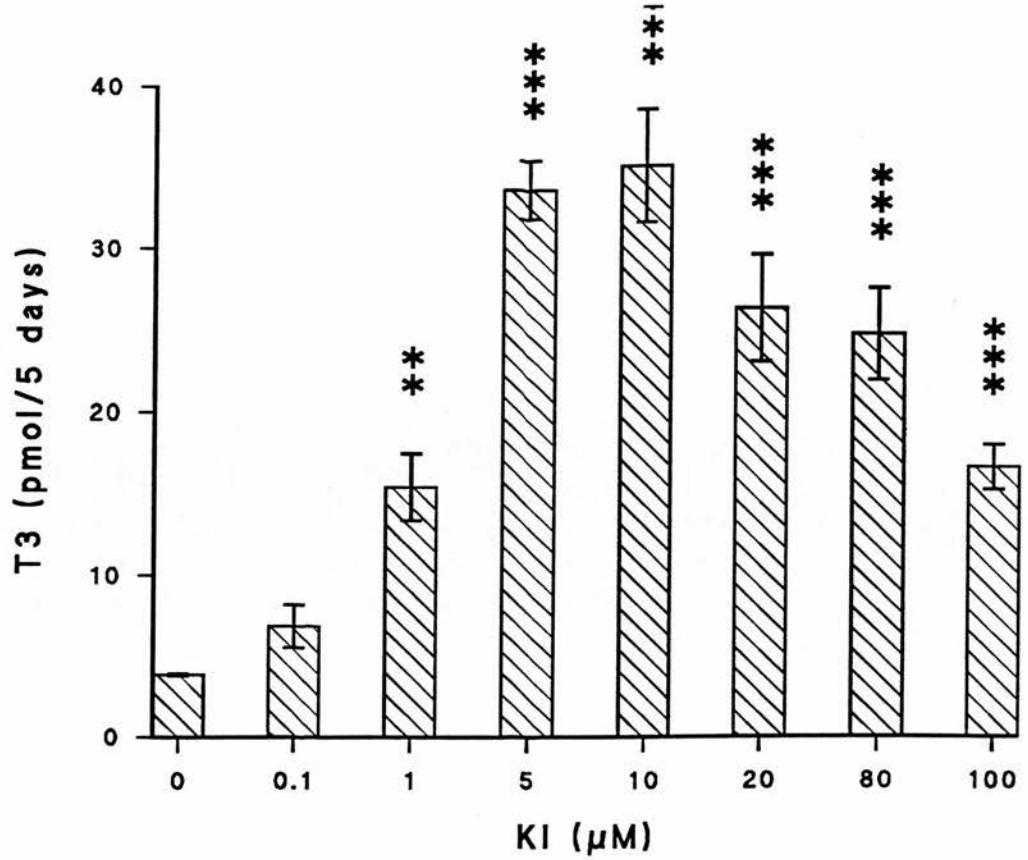

T4 (pmol/5 days)
15
10
5
0
**
**
**
0
25
50
100
200
500
1000
10000
TSH (mU/L)

Figure 3d : Effect of increasing doses of KI on thyroid hormone production from sheep thyrocytes grown in primary culture for 5 days in the presence of 1 U/L TSH. The accumulated medium content of T_3 and T_4 was measured and the data expressed as mean ± SEM of triplicate wells in a representative experiment carried out on at least three occasions. KI concentrations in excess of 1 μM gave significant increases in T_3 and T_4 accumulation compared to the control (- KI). At a concentrations of 100μM, KI caused a significant decrease in both T_3 and T_4 accumulation, when compared to 10μM ($p < 0.01$).


T3 (pmol/5 days)
40
30
20
10
0
0
0.1
1
5
10
20
80
100
KI (μM)

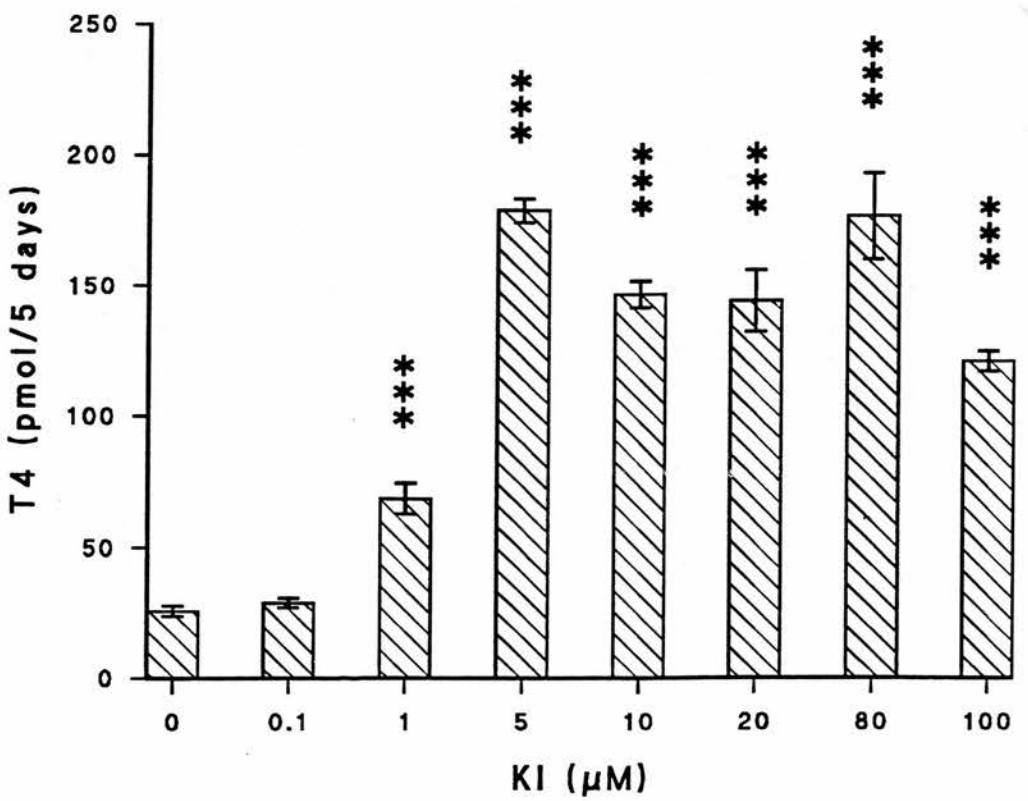
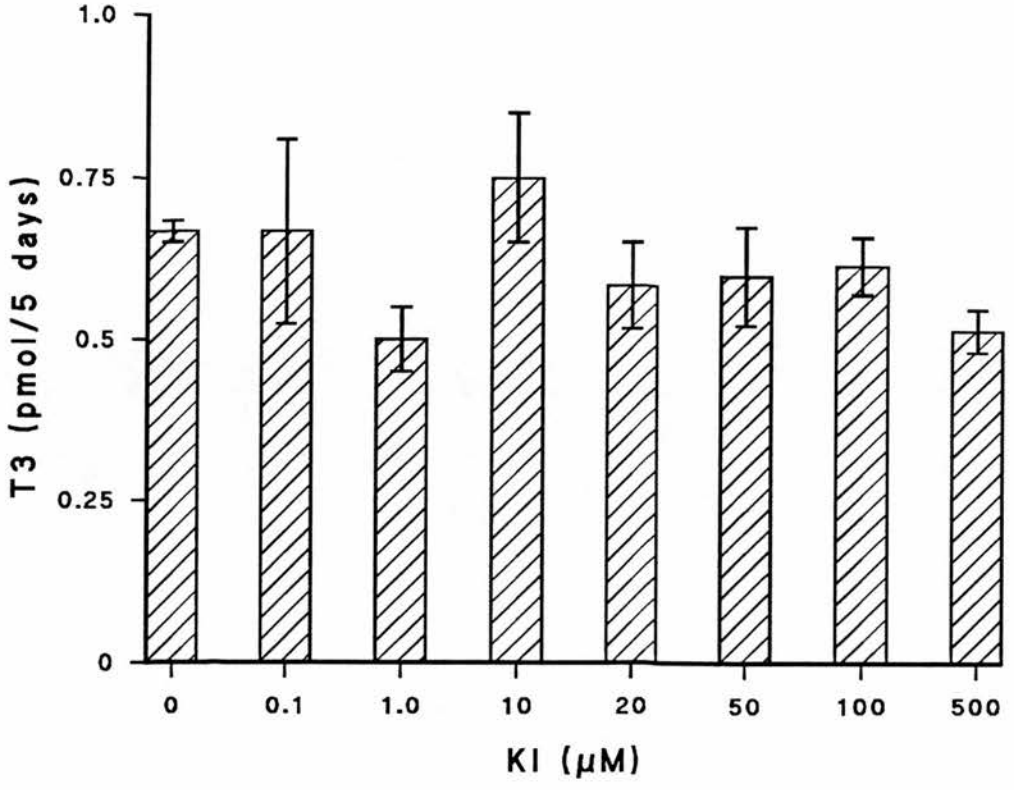

T4 (pmol/5 days)
250
200
150
100
50
0
0
0.1
1
5
10
20
80
100
KI (μM)

Figure 3e : Effect of increasing doses of KI on thyroid hormone production from human thyrocytes grown in primary culture for 5 days in the presence of 1 U/L TSH. The accumulated medium content of T_3 and T_4 was measured and the data expressed as mean ± SEM of triplicate wells in a representative experiment carried out on at least three occasions. No significant changes in T_3 and T_4 accumulation were observed.


1.0
0.75
0.5
0.25
0
T3 (pmol/5 days)
0
0.1
1.0
10
20
50
100
500
KI (μM)

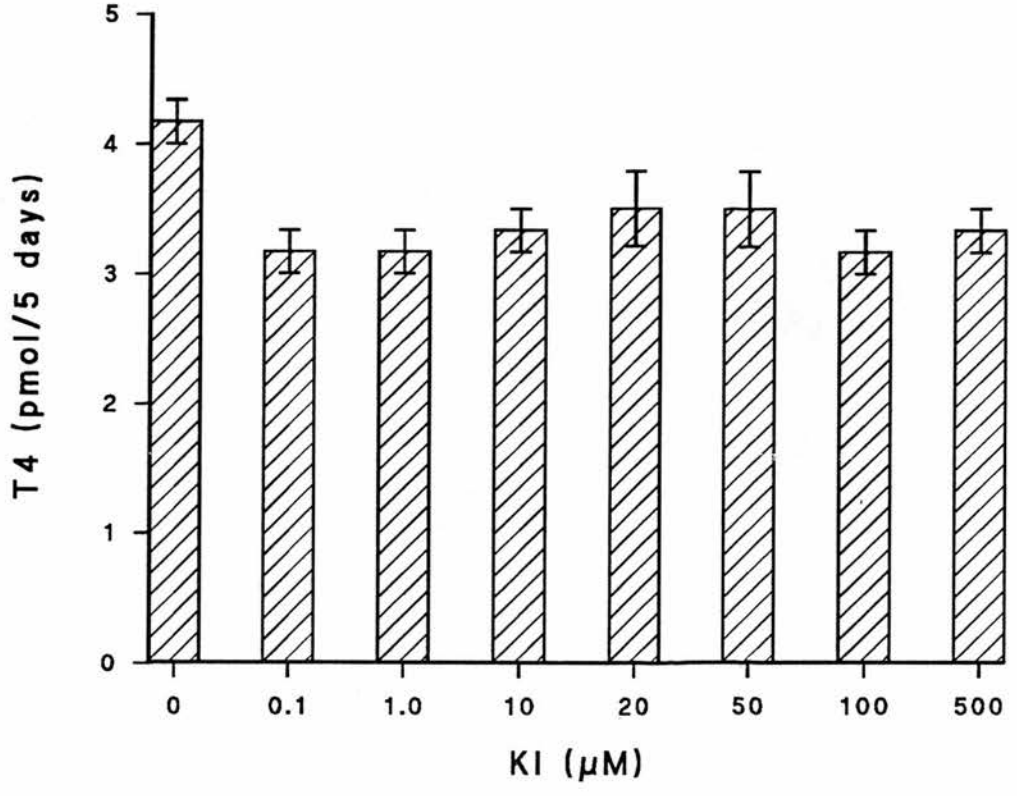
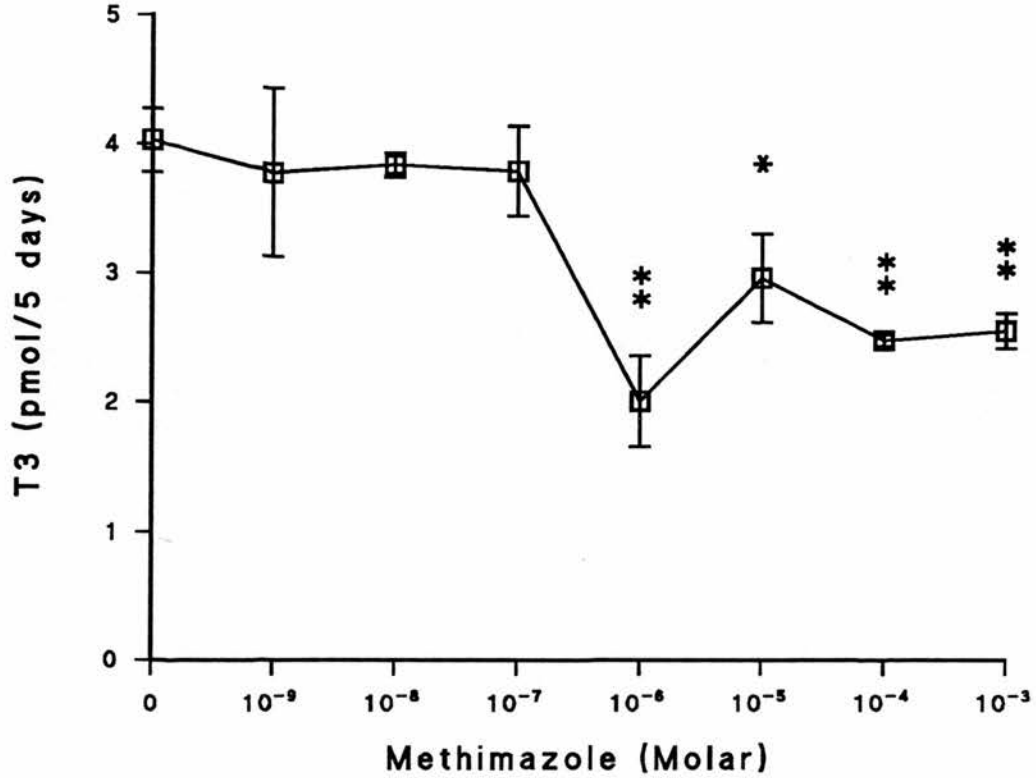

5
4
3
2
1
0
T4 (pmol/5 days)
0
0.1
1.0
10
20
50
100
500
KI (μM)

Figure 3f : Effect of 5 days exposure to methimazole on thyroid hormone production from sheep thyrocytes grown in primary culture. The accumulated medium content of T_3 (□) and T_4 (Δ) was measured and the data expressed as mean ± SEM of triplicate wells in a representative experiment carried out on at least three occasions. Methimazole at concentrations above 10^{-7}M caused significant decreases in T_3 and T_4 accumulation when compared to the control (i.e. - methimazole).


T3 (pmol/5 days)
5
4
3
2
1
0
0
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
Methimazole (Molar)

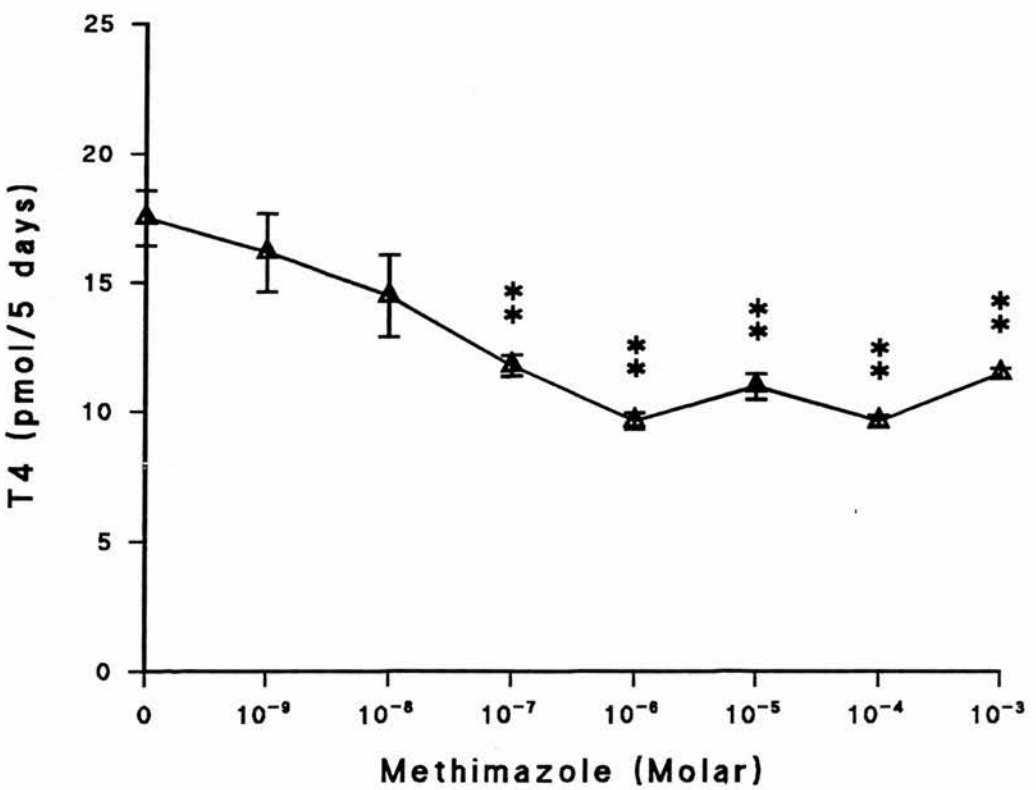
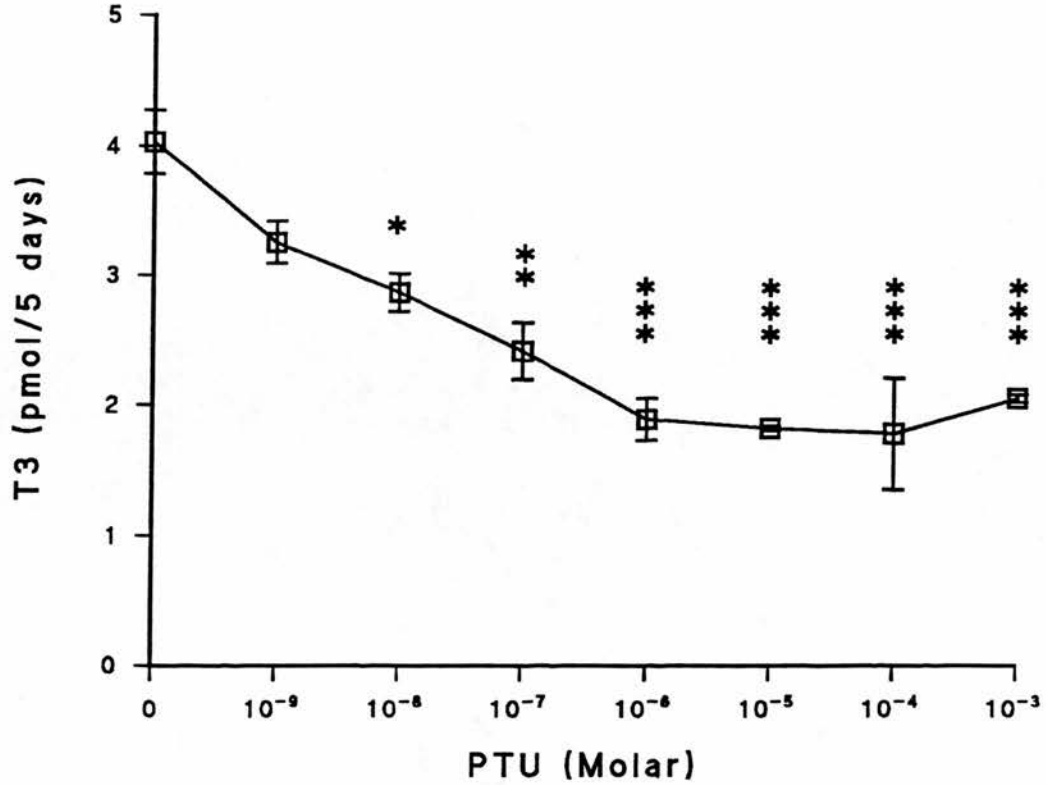

T4 (pmol/5 days)
25
20
15
10
5
0
0
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
Methimazole (Molar)

Figure 3g : Effect of 5 days exposure to PTU on thyroid hormone production from sheep thyrocytes grown in primary culture. The accumulated medium content of T_3 (□) and T_4 (Δ) was measured and the data expressed as mean ± SEM of triplicate wells in a representative experiment carried out on at least three occasions. PTU in excess of 10^{-9}M caused significant decreases in both T_3 and T_4 accumulation when compared to the control (i.e. - PTU).


T3 (pmol/5 days)
5
4
3
2
1
0
0
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
PTU (Molar)

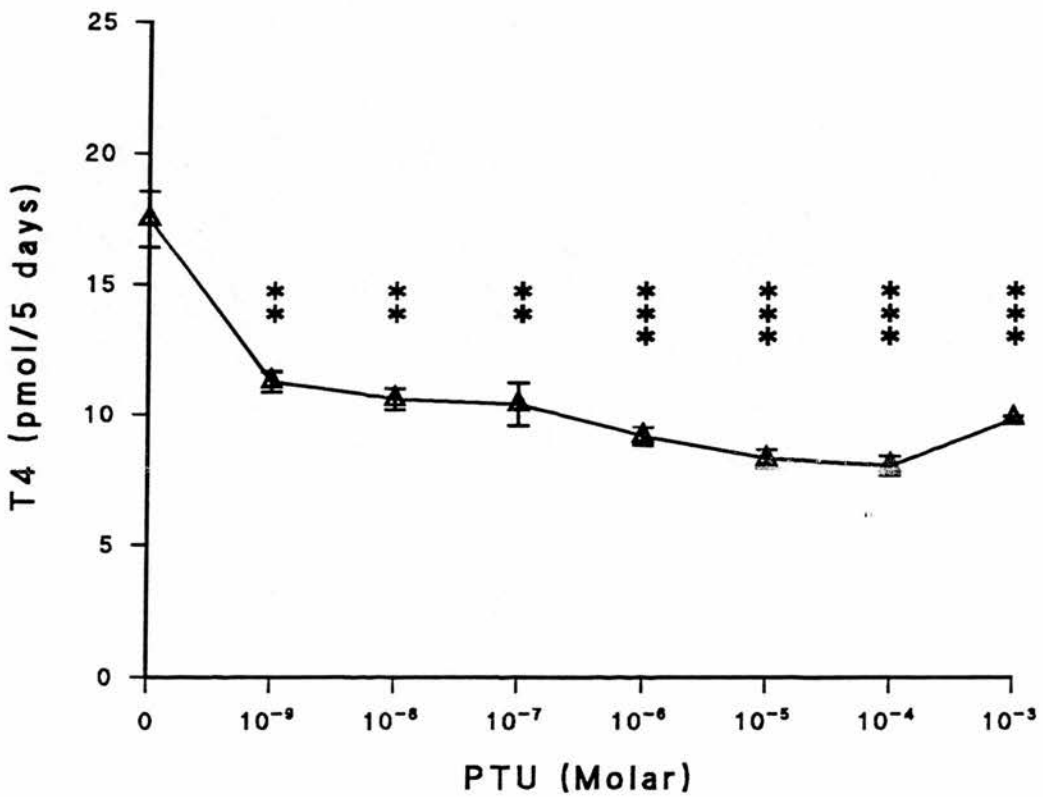
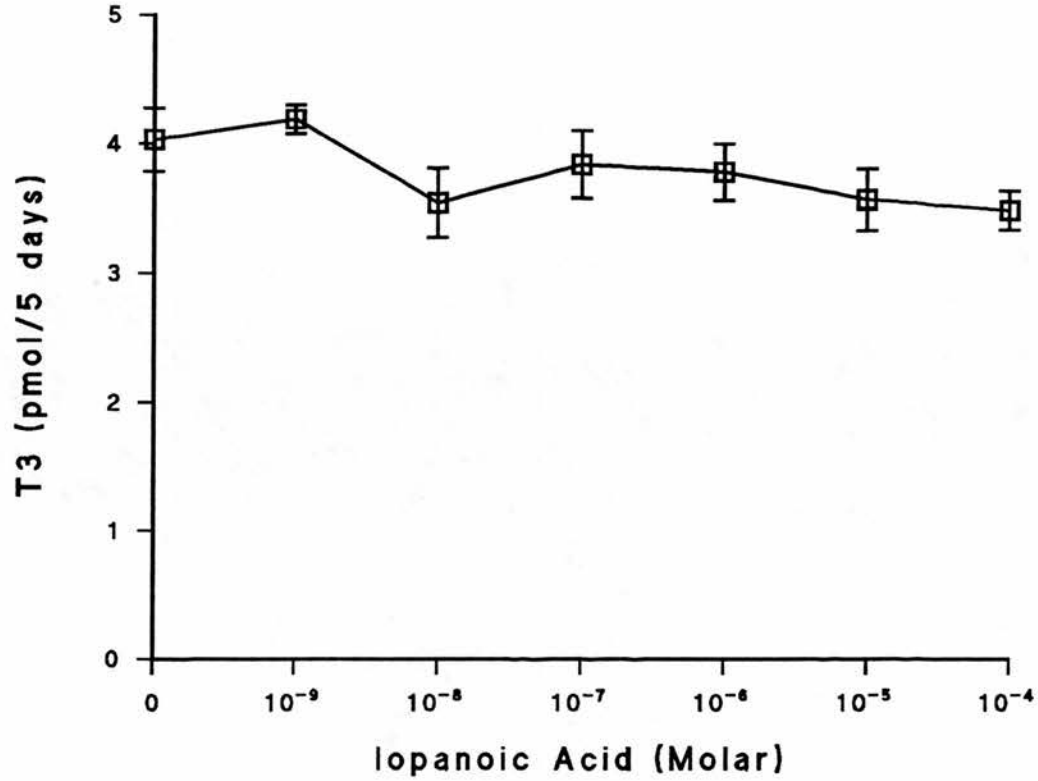

T4 (pmol/5 days)
25
20
15
10
5
0
0
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
PTU (Molar)

Figure 3h : Effect of 5 days exposure to IPA on thyroid hormone production from sheep thyrocytes grown in primary culture. The accumulated medium content of T_3 (□) and T_4 (Δ) was measured and the data expressed as mean ± SEM of triplicate wells in a representative experiment carried out on at least three occasions. IPA at a concentration of 10^{-4}M caused a significant increase in T_4 accumulation compared to the control (i.e. - IPA).


T3 (pmol/5 days)
5
4
3
2
1
0
0
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
Iopanoic Acid (Molar)

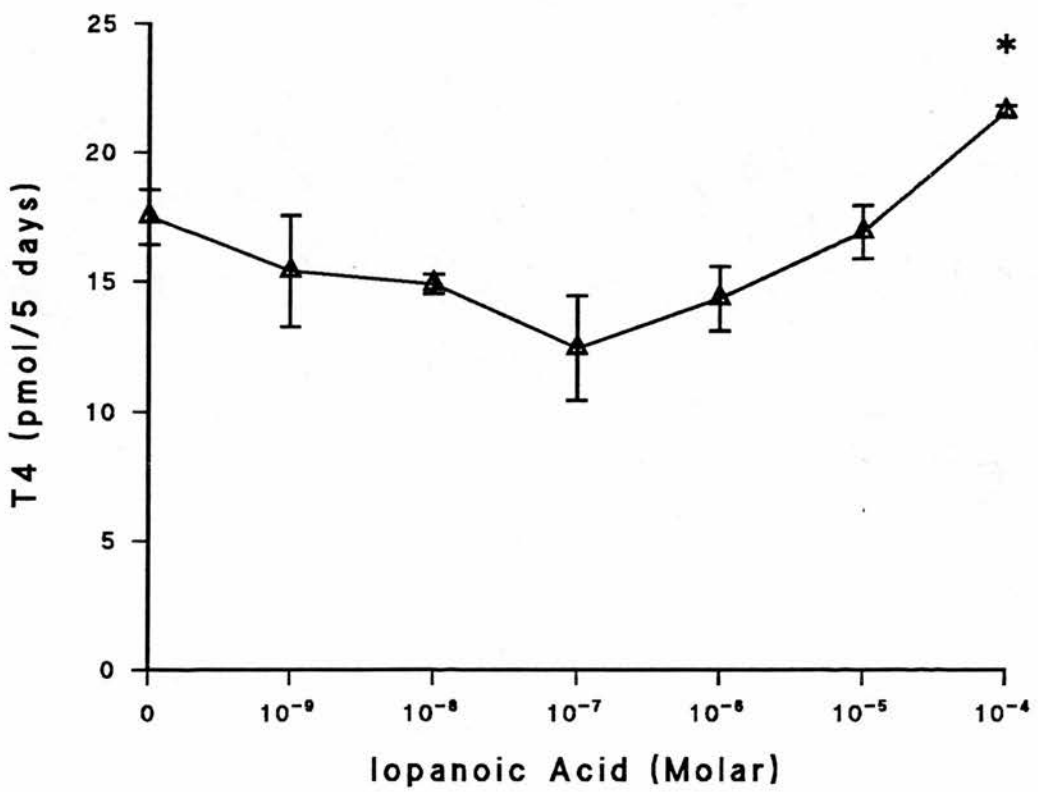
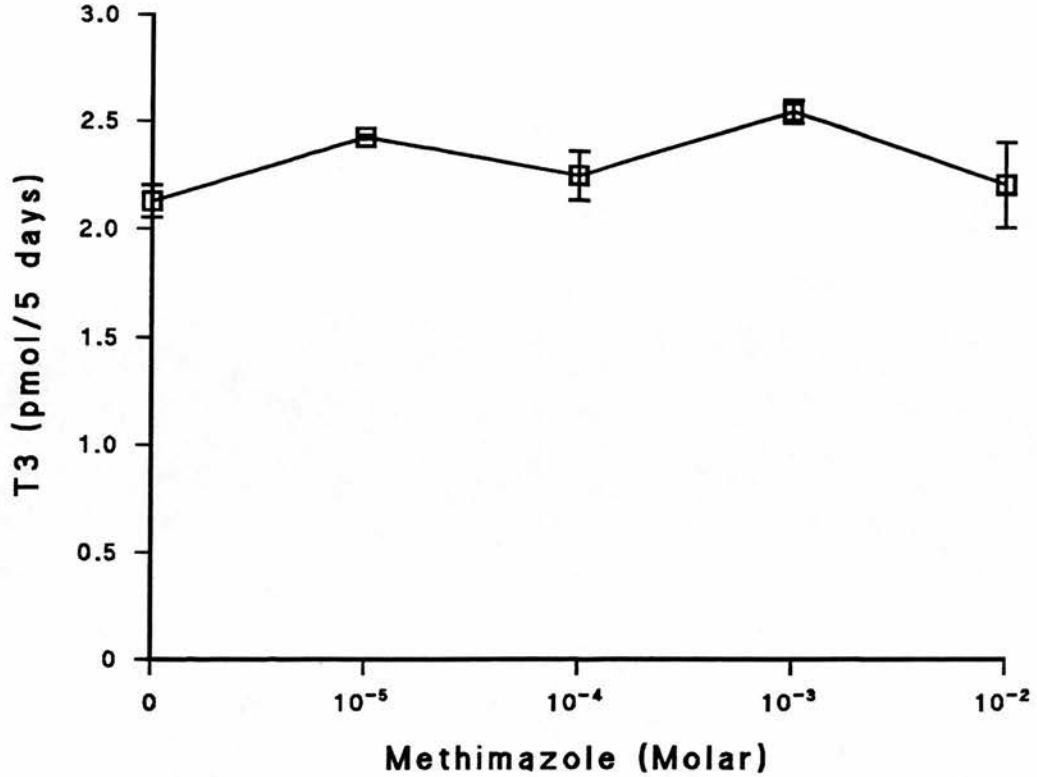

T4 (pmol/5 days)
25
20
15
10
5
0
*
0
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
Iopanoic Acid (Molar)

Figure 3i : Effect of 5 days exposure to methimazole on thyroid hormone production from human thyrocytes grown in primary culture. The accumulated medium content of T_3 (□) and T_4 (Δ) was measured and the data expressed as mean ± SEM of triplicate wells in a representative experiment carried out on at least three occasions. Methimazole at concentrations in excess of 10^{-4}M gave a significant decrease in T_4 accumulation when compared to the control (i.e. - methimazole).


3.0
2.5
2.0
1.5
1.0
0.5
0
T3 (pmol/5 days)
0
10⁻⁵
10⁻⁴
10⁻³
10⁻²
Methimazole (Molar)

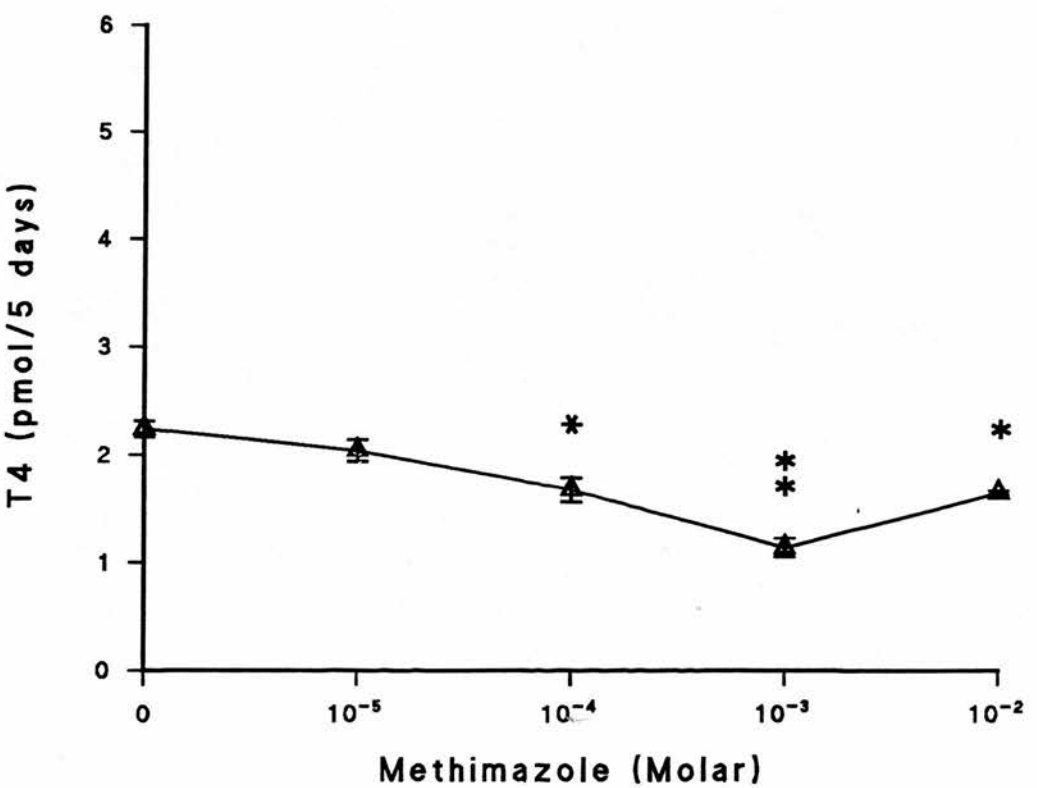
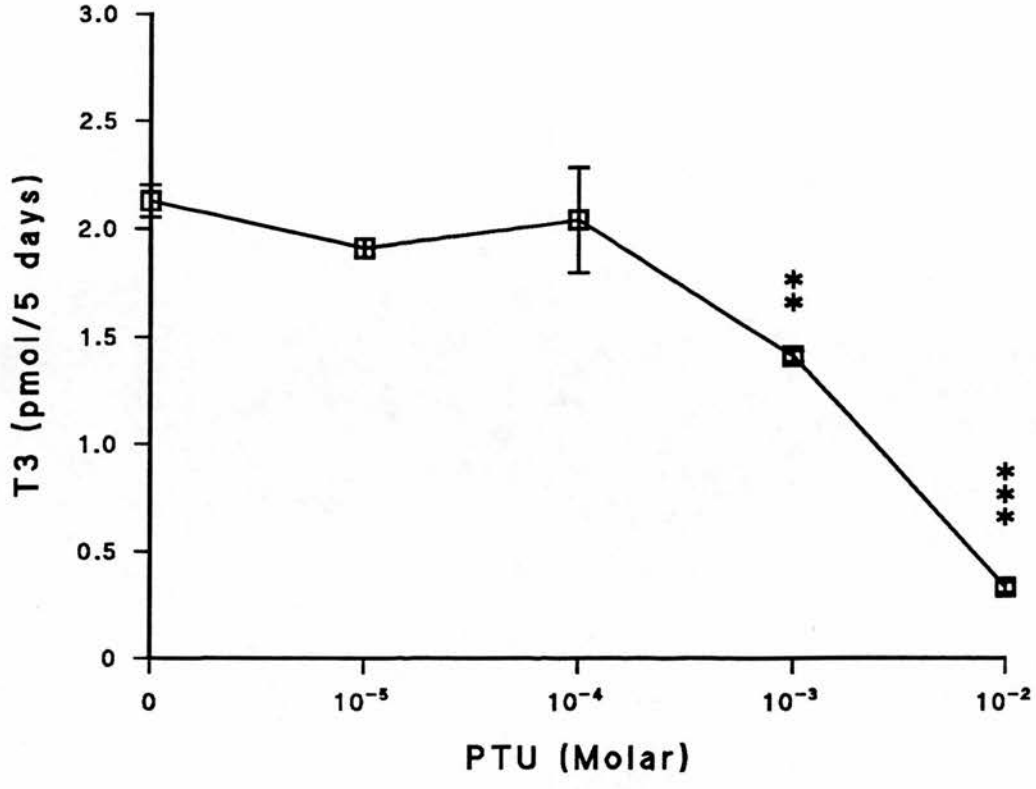

6
5
4
3
2
1
0
T4 (pmol/5 days)
0
10⁻⁵
10⁻⁴
10⁻³
10⁻²
Methimazole (Molar)

Figure 3j : Effect of 5 days exposure to PTU on thyroid hormone production from human thyrocytes grown in primary culture. The accumulated medium content of T_3 (□) and T_4 (Δ) was measured and the data expressed as mean ± SEM of triplicate wells in a representative experiment carried out on at least three occasions. Significant decreases in T_3 accumulation occurred at PTU concentrations above 10^{-3}M, while a significant increase in T_4 accumulation occurred at 10^{-2}M when compared to the control (i.e. - PTU).


3.0
2.5
2.0
1.5
1.0
0.5
0
T3 (pmol/5 days)
**
***
0
10⁻⁵
10⁻⁴
10⁻³
10⁻²
PTU (Molar)

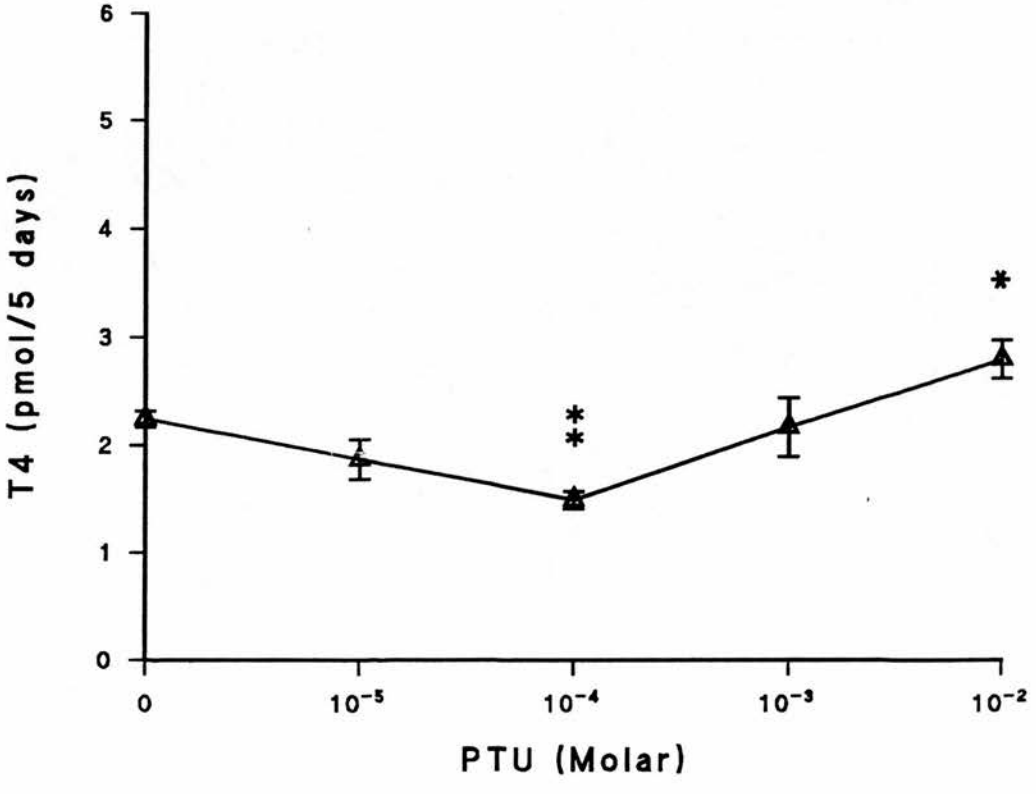
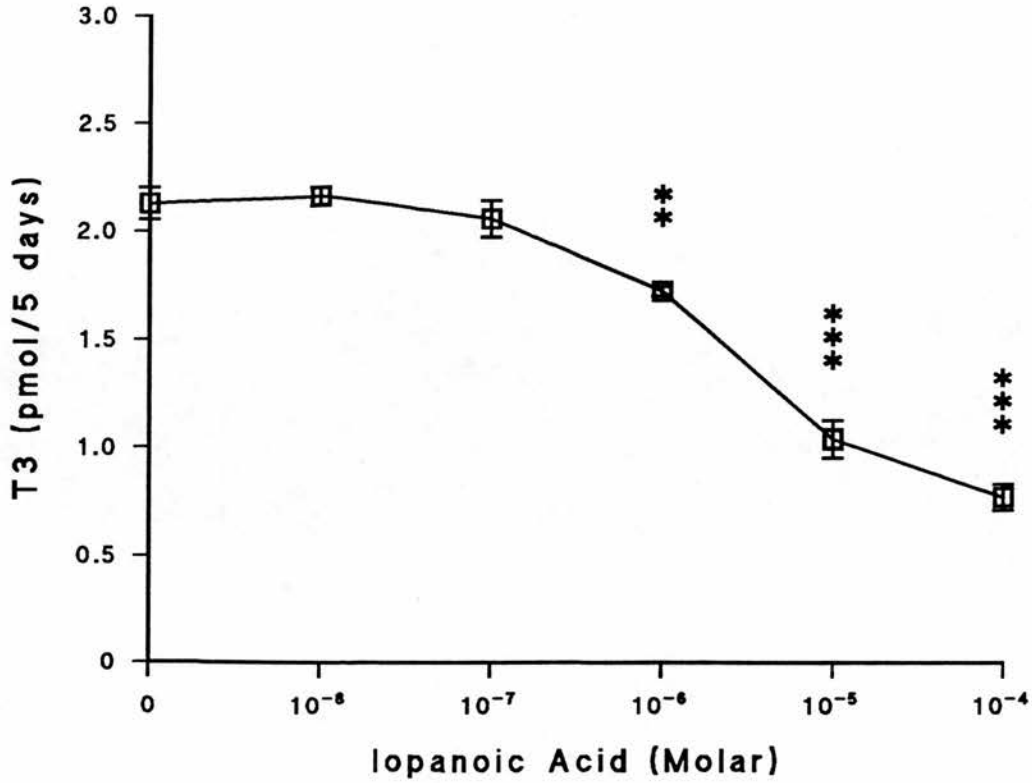
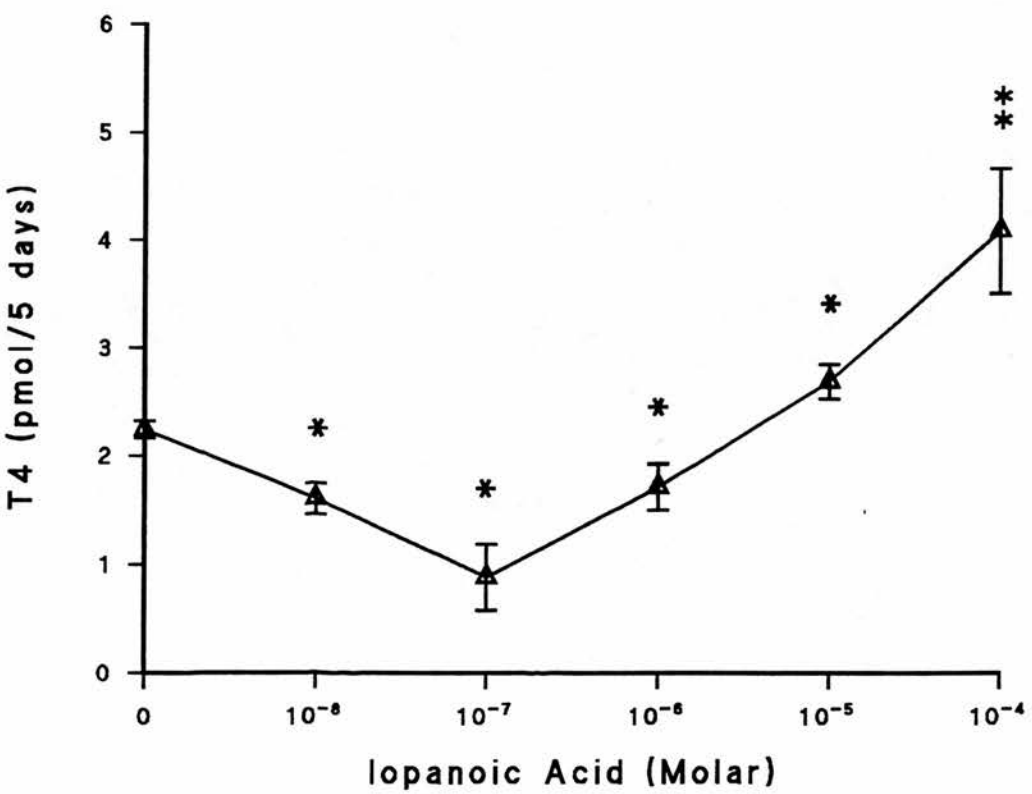

6
5
4
3
2
1
0
T4 (pmol/5 days)
*
**
0
10⁻⁵
10⁻⁴
10⁻³
10⁻²
PTU (Molar)

Figure 3k : Effect of 5 days exposure to IPA on thyroid hormone production from human thyrocytes grown in primary culture. The accumulated medium content of T_3 (□) and T_4 (Δ) was measured and the data expressed as mean ± SEM of triplicate wells in a representative experiment carried out on at least three occasions. Significant decreases in T_3 accumulation occurred at IPA concentrations above 10^{-6}M, while significant changes in T_4 accumulation occurred above 10^{-8}M when compared to the control (i.e. - IPA).


3.0
2.5
2.0
1.5
1.0
0.5
0
T3 (pmol/5 days)
0
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
Iopanoic Acid (Molar)


6
5
4
3
2
1
0
T4 (pmol/5 days)
0
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
Iopanoic Acid (Molar)

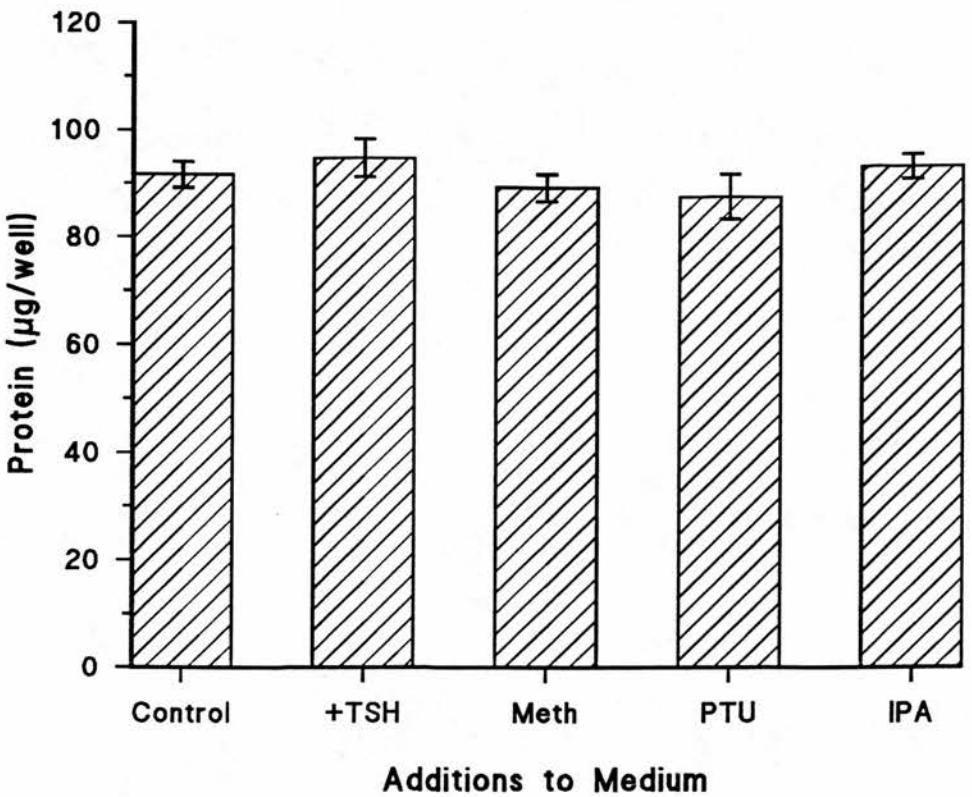
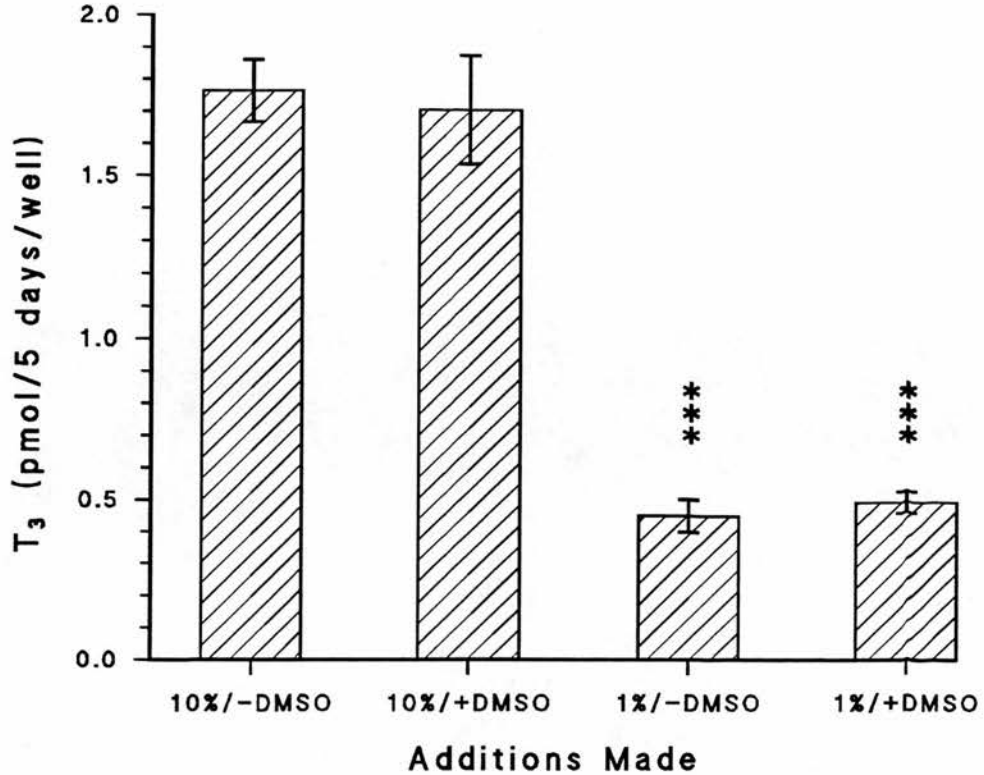
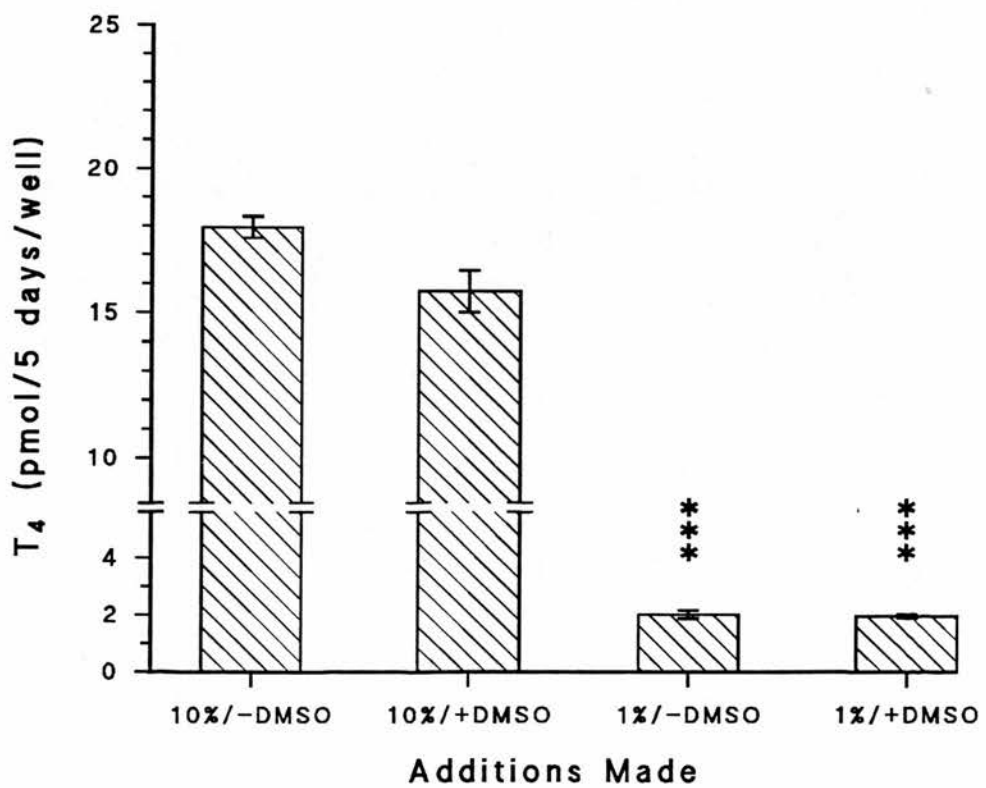


Figure 3l : Effect of 5 days exposure to TSH (1 U/L), methimazole (10^{-3}M), PTU (10^{-3}) or IPA (10^{-4}M) on the protein content of human thyrocytes grown in primary culture. The total protein content of each well was measured and the data expressed as mean ± SEM of triplicate wells in a representative experiment carried out on at least three occasions. No significant changes in the protein content of the wells occurred.

Figure 3m : Effect of 5 days exposure to DMSO (1.7%) on thyroid hormone production from human thyrocytes grown in primary culture with either 1% or 10% CPSR-5 (fetal calf serum) present. The accumulated medium content of T_3 (□) and T_4 (Δ) was measured and the data expressed as mean ± SEM of triplicate wells in a representative experiment carried out on at least three occasions. The presence of DMSO caused no significant change in T_3 and T_4 accumulation, though the lower concentration of CPSR-5 did cause a significant decrease in the accumulation of T_3 and T_4.


2.0
1.5
1.0
0.5
0.0
T_3 (pmol/5 days/well)
***
***
10%/-DMSO
10%/+DMSO
1%/-DMSO
1%/+DMSO
Additions Made


25
20
15
10
4
2
0
T_4 (pmol/5 days/well)
***
***
10%/-DMSO
10%/+DMSO
1%/-DMSO
1%/+DMSO
Additions Made

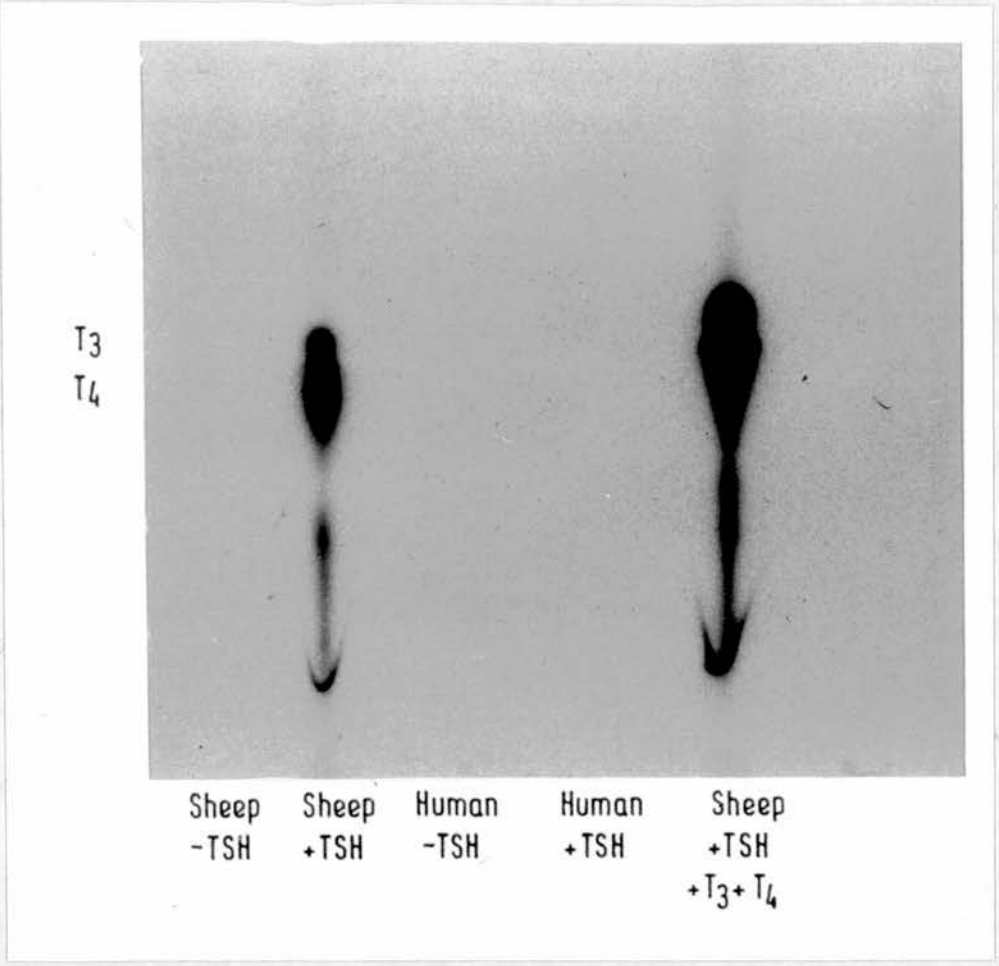


Figure 3n : A thin-layer chromatography of radiolabelled tri-iodothyronine and thyroxine, immunoprecipitated from culture medium following a 5 day incubation of sheep and human thyrocytes with $Na^{125}I$ in the presence and absence of TSH (1 U/L). The TLC shows incorporation of ^{125}I into T_3 and T_4 in sheep, but not human thyrocytes in response to TSH stimulation. Lane 5 contains similar amounts of the material run in Lane 2 with the addition of $[^{125}I]$-T_3 and $[^{125}I]$-T_4 to act as internal standards.

3.03 : Discussion

In the absence of TSH, the production of both thyroid hormones by sheep thyrocytes was very low (figure 3a). Addition of TSH alone caused a significant increase in the amount of both thyroid hormones, though maximum production required both TSH and KI. In my experiments maximum concentrations of T_3 and T_4 were observed after 8 days, however the levels of T_3 and T_4 could well have continued to rise. Obviously, some of this effect might be due to growth and proliferation of the thyrocytes, however it is unlikely that a 10-fold increase could be the result of just cell proliferation. In subsequent experiments, a 5 day incubation was chosen as this length of time allows significant levels of both thyroid hormones to be produced and convenient timing of the experiments.

The production of T_3 and T_4 by sheep thyrocytes was clearly dependent on both the TSH and KI concentration, with maximum production of both thyroid hormones at 1 U/L and 10 μM respectively (figures 3b & 3d). At concentrations of TSH and KI above the optimum values (> 1 U/L TSH and > 10 μM KI), significant decreases in the production of both thyroid hormones were observed. Other workers have shown using human thyroid slices and human thyrocytes in primary culture that high TSH concentrations bring about the stimulation of the Ca^{2+}-phosphatidylinositol (Ca^{2+}-PI) cascade and inhibition of the cAMP cascade (Laurent *et al,* 1987; Laurent *et al,* 1989; Raspe *et al,* 1991b; Maenhaut *et al,* 1990; Raspe and Dumont, 1992). The Ca^{2+}-PI cascade is believed to control the synthesis of T_3 and T_4 via control of the rate limiting steps of thyroid hormone synthesis (iodine and hydrogen peroxide supply to thyroperoxidase), while the cAMP cascade mediates release of T_3 and T_4 from thyroglobulin (Raspe and Dumont, 1992). Consequently, it is possible that excess TSH might be able to increase synthesis of both thyroid hormones in sheep thyrocytes, but reduce the amount of T_3 and T_4 secreted into the overlying medium.

The observed decreases in the T_3 and T_4 content of the medium at KI concentrations in excess of 10 μM are supported by Becks *et al*, who demonstrated that "excessive" concentrations of iodide ($\geq$ 10 μM) reduce the amount of T_3 and T_4 secreted by sheep thyrocytes (Becks *et al,* 1987). Several other workers have also shown inhibition of thyroid hormone secretion by iodide (Mashita *et al,* 1982; Laurberg, 1982), as well as inhibition of iodine transport, organification and thyroid hormone synthesis (Wolff and Chaikoff, 1948; Grollman *et al,* 1986; Shimoda *et al,* 1966; Bagchi *et al,* 1977; Braverman and Ingbar, 1963). This inhibition of thyroid hormone secretion by high levels of iodide has been postulated to occur by an organified iodine intermediate (X~I), based on the observations that relief from the effects of excess iodide are found on administration of drugs which inhibit iodine organification (Van Sande and Dumont, 1973). This X~I species is believed to inhibit adenylate cyclase, possibly via stimulating an inhibitory GTP-binding transduction protein (G_i) (Van Sande and Dumont, 1973; Cochaux *et al,* 1987; Corvilain *et al,* 1988; Raspe and Dumont, 1992). In addition to thyroid hormone secretion, the X~I intermediate has also been shown to inhibit glucose transport, uridine incorporation and hydrogen peroxide generation (Corvilain *et al,* 1988).

The accumulation of T_3 and T_4 in the medium bathing the sheep thyrocytes was inhibited by methimazole and PTU (figures 3f & 3g). Methimazole and PTU both inhibit a number of steps in the *de novo* synthesis of T_3 and T_4 including oxidation of iodide, iodination of tyrosyl residues to produce mono/di-iodotyrosines and the coupling of these iodotyrosyl residues on thyroglobulin to make the thyroid hormones. In addition, when ^{125}I was added to the growth medium, incorporation of ^{125}I into both T_3 and T_4 occurred, but only in the presence of TSH (figure 3n).

These data clearly establish that sheep thyrocytes are able to produce both T_3 and T_4 by *de novo* synthesis under these culture conditions. However, in the sheep, IID-I can have no significant role in thyroidal T_3 production since the thyroid of this species lacks

notable levels of ID-I expression (Section 4.02a,b). In support of this, the addition of iopanoic acid (a specific ID-I inhibitor) had no effect on T_3 production by sheep thyrocytes (figure 3h).

Accumulation of T_3 in the medium bathing the human thyrocytes occurred in a dose-dependent manner with respect to TSH, with maximum concentrations of T_3 measured at 1 U/L TSH (figure 3c). In contrast, the T_4 content of the medium was reduced as the concentration of TSH exceeded 0.5 U/L, with the largest reduction measured at 1 U/L TSH. However unlike sheep thyrocytes, the T_3 and T_4 accumulating in the medium bathing the human thyrocytes appeared to be independent of the KI concentration (figure 3e). Moveover, the human thyrocytes also failed to incorporate ^{125}I into T_3 or T_4, even under TSH stimulation (figure 3n). Finally, T_3 production by the human thyrocytes was clearly inhibited in a dose-dependent manner by IPA and PTU, but not by methimazole.

These observations are consistent with T_3 production by human thyrocytes arising from deiodination of endogenous T_4 present within the thyrocytes, with little or no *de novo* synthesis of T_4 or T_3. These data support the findings of other workers, who showed that human thyrocytes grown in culture are unable to concentrate and organify free iodide even after several days in culture (Bidey *et al,* 1977; Ollis *et al,* 1985). These workers showed that T_3 continued to accumulate in the overlying medium, and Ollis *et al* proposed that human thyrocytes in primary culture release T_3 from intracellular stores when stimulated with TSH, rather than by synthesizing new T_3. A significant component of this secreted T_3 may well arise from 5'-deiodination of T_4 as human thyrocytes express significant levels of ID-I, in contrast to sheep thyrocytes (Section 4.02a,b). The TSH-dependent production of T_3 by human thyrocytes can be explained on the basis of the TSH-responsiveness of ID-I, most probably mediated by cyclic AMP (Erickson *et al,* 1982; Ishii *et al,* 1983).

Other workers have proposed that T_3 production from isolated human thyrocytes in primary culture arises from *de novo* synthesis (Kraiem *et al,* 1988). These workers based their conclusions on the observation that PTU inhibited T_3 production, an observation confirmed here (figure 3j). However, since PTU inhibits *de novo* synthesis of thyroid hormones and the deiodination of T_4, the inhibition of T_3 accumulation in the medium by PTU clearly can not distinguish between these two sources of T_3. Kraiem *et al* also reported that methimazole inhibited T_3 production, though no data in support of this statement was published. Furthermore, the effects of IPA were not examined in their study. We found no inhibitory effects of methimazole on T_3 production, even at concentrations as high as 10 mM.

Sato *et al* using a culture system of isolated human follicles demonstrated that the presence of DMSO (1.7%) enhanced the effects of TSH on a number of thyroid functions (iodine trapping, organification and release of T_3 and T_4) (Sato *et al,* 1988). In their experiments, lowering the concentrations of fetal calf serum from 10% to 1% resulted in further enhancement of these markers of thyroid function. They postulated that this enhancement was possibly because DMSO prevented dedifferentiation of the thyrocytes, and as a consequence allowed *de novo* synthesis of thyroid hormones. In addition, they stated that high concentrations of fetal calf serum (10%) caused deterioration of thyroid function, but could not explain this observation. I have tried on numerous occasions to repeat the findings of Sato *et al* with respect to *de novo* synthesis of T_3 and T_4 by human thyrocytes, but have not observed any effect of DMSO in the human thyrocyte system (figure 3m). Furthermore, I found that a reduced concentration of fetal calf serum (CPSR-5) results in a reduction in the amount of T_3 and T_4 which accumulates in the overlying medium. The reason behind these observed differences may lie in differences between the thyrocyte preparations and other experimental methods used. Sato *et al* used follicular preparations plated out in 24 well plates, previously treated with 0.5% agarose. The

presence of agarose prevented cell attachment and hence the thyrocytes remained in "suspension" culture. In contrast, I have used a preparation with few follicles present, and allowed the thyrocytes to attach to the cell culture plates. In addition, I used human TSH at 1 U/L as compared to Sato *et al* who used 0.1 U/L of bovine TSH. Although these differences in method are not great, they remain the most likely cause of the differences described. Indeed other workers has demonstrated that the thyrocytes have to be arranged in follicles for the secretion of iodinated thyroglobulin (Lissitzky *et al,* 1971; Winand *et al,* 1976; Wadeleux *et al,* 1978). However, why the human thyrocytes in this study can notsynthesisethyroid hormones even when organised into TSH-induced follicles remains to be explained.

In summary under the culture conditions used, sheep thyrocytes produce both T_3 and T_4 as a result of *de novo* synthesis. The formation of T_3 by sheep thyrocytes cannot be explained by deiodination of T_4 as this tissue lacks significant amounts of ID-I (see Section 4). In contrast, human thyrocytes produce T_3 as a result of deiodination of endogenous T_4, with no significant *de novo* synthesis. Consequently in man, ID-I may well contribute significantly to T_3 secretion, particularly under conditions which giving rise to stimulation of the TSH receptor such as iodine deficiency or Graves' disease. As the sheep lacks thyroidal ID-I, this mechanism to maintain plasma T_3 levels is unavailable.

Section 4 : THYROIDAL ID-I EXPRESSION IN MAN AND ANIMALS

Data presented in Section 3 and the findings of numerous other workers (Green, 1978; Laurberg, 1980; Laurberg, 1984; Pazos-Moura *et al,* 1991) suggest that significant amounts of T_3 can be produced as a result of 5'-deiodination of T_4 in the thyroid, particularly under conditions which give rise to high levels of TSH receptor stimulation, such as iodine deficiency and Graves' disease. The possible importance of ID-I in producing T_3 is further supported by the finding of a number of other workers who have shown that the thyroids of humans, dogs and rats contain an ID-I which is responsive to TSH, via stimulation of the cyclic AMP pathway (Erickson *et al,* 1982; Wu, 1983; Ishii *et al,* 1983; Silva *et al,* 1984).

In this section I study the levels of ID-I expression in the liver and thyroid of a range of animal species. I then proceed to investigate the effects of TSH and second messengers on ID-I activity and expression in human thyrocytes grown in primary culture. In addition, I compare the levels of activity and expression of ID-I found in thyroid tissue from patients with multinodular goitre and Graves' disease.

4.01 : Methods

a) Species differences in hepatic and thyroidal ID-I activity

The livers and thyroids of the 11 species studied (sheep, llama, man, cattle, deer, pig, goat, rat, guinea-pig, mouse and rabbit) were removed into liquid nitrogen within an hour of death. Subsequently the tissues were thawed, homogenised in ID-I assay buffer to obtain a 20% (w/v) homogenate and centrifuged for 15 min at 300g before being assayed for ID-I activity as described in Section 2.05a.

b) Species differences in the affinity labelling of thyroidal homogenates

The thyroids and livers from 8 species (sheep, llama, man, cattle, deer, pig, goat and rat) were thawed and homogenised in labelling buffer (50 mmol/L Tris/HCl buffer containing 3 mmol/L EDTA and 3 mmol/L DTT, pH 7.4) to obtain a 20% (w/v) homogenate. The homogenates were then centrifuged at 300g for 15 min and diluted to a protein concentration of 1 g/L. The homogenates were labelled with n-bromoacetyl-[^{125}I]-rT_3 as described in Section 2.08. The proteins were separated by SDS-PAGE and the gels autoradiographed as described in Sections 2.09 and 2.10. The hepatic homogenates were labelled in the presence of PTU (500 μM) and rT_3 (10 μM). PTU and rT_3 selectively inhibit the binding to, and affinity labelling of ID-I by n-bromoacetyl-[^{125}I]-rT_3. This work was performed by Dr. J.R. Arthur and F. Nicol as part of a collaborative study.

c) Effect of TSH on thyroidal ID-I activity in human thyrocytes

Human thyrocytes were prepared as described in Section 2.03. After 24 h in monolayer culture the medium was removed from the thyrocytes and the cells washed twice with 1 ml of EBS before 1 ml of fresh growth medium (DMEM/10% CPSR-5) was added. To wells in triplicate a range of TSH doses was added in the presence of 100 nmol/L selenium (Se, added as sodium selenite). After 5 days the thyrocytes were washed again and the contents of each well removed in 1 ml of ID-I assay buffer after using a rubber policeman to detach the thyrocytes from the well. The ID-I activity and protein content of the thyrocyte sonicates were then determined as described in Sections 2.06 and 2.07.

d) Effect on thyroidal ID-I activity of activating the different second messenger systems found in human thyrocytes

Human thyrocytes were prepared and washed as described above. To wells in triplicate, various compounds were added in the presence and absence of TSH (1 U/L). These

included i) 8-bromoadenosine 3':5'-cyclic monophosphate (8-bromo-cAMP, 10^{-4}M), ii) the phorbol ester, phorbol 12-myristate 13-acetate (PMA, 10^{-6}M), iii) the calcium ionophore, A23187 (10^{-6}M), iv) PMA and A23187 together or v) 1% dimethylsulphoxide (DMSO, added as a control for PMA and A23187 which were added in 1% DMSO). After 5 days the thyrocytes were washed and removed from the wells as described in Section 4.01c and assayed for ID-I activity and protein (Section 2.06 and 2.07).

e) Changes in the affinity labelling of human thyrocytes after activation of the different second messenger systems

Human thyrocytes were prepared as described in Section 2.03 and plated out in 75cm^2 flasks at a density of 10 million cells per flask in 20 ml of growth medium (DMEM/10% CPSR-5). After 24 h the medium was removed and the cells washed twice with 20 ml of EBS before 20 ml of fresh growth medium was added. To half of the cells, PMA and A23187 were added, both at a concentration of 1 μM in the presence or absence of TSH (1 U/L). The remaining cells were grown in the absence of PMA/A23187 but with the presence or absence of 1 U/L TSH.

After 5 days incubation the medium was removed and the cells washed twice as before with EBS. The cells were then washed with 20 ml of 0.02% (w/v) EDTA in Ca^{2+}/Mg^{2+}-free EBS, before being incubated with gentle agitation for 5-10 min with 20 ml of 0.25% (w/v) Trypsin, 0.02% (w/v) EDTA in Ca^{2+}/Mg^{2+}-free EBS to remove the cells from the flask. The released cells were then diluted 2-fold with DMEM/10% CPSR-5 before being pelleted by centrifugation at 125 g for 15 min and washed with 20 ml of EBS. Following re-centrifugation another wash and centrifugation was performed before the cell pellet was frozen at -70°C. Subsequently the cell pellets were sonicated in 1 ml of labelling buffer, diluted to a protein concentration of 1 g/L and affinity labelled as described in Section 2.08.

In light of the results obtained using the affinity label (i.e changes in intensity of the 44.5 and 45 kDa affinity labelled bands), creatine kinase (CK) activity was measured in the cell sonicates on a Kodak Ektachem 700 analyzer (assay kindly performed by Dr. A.F. Howie). The activity of creatine kinase was measured using the method recommended by the Scandinavian Committee on Enzymes (*Scand.J.Clin.Lab.Invest*, 1976 : 36,711). The method consisted of a creatine kinase - glycerol kinase - L-α-glycerophosphate oxidase - peroxidase linked enzyme assay system to produce a coloured dye, the concentration of which was assayed by absorbance spectrophotometry. The reaction sequence is as follows, with the enzymes and co-factors for each reaction listed in brackets :-

a) creatine phosphate + ADP → creatine + ATP (CK, N-acetylcysteine, Mg^{2+})

b) ATP + glycerol → α-glycerophoshate + ATP (glycerol kinase)

c) α-glycerophosphate + O_2 → dihydroxyacetone phosphate + H_2O_2 (L-α-glycerophosphate oxidase)

d) H_2O_2 + leuco dye precursor → coloured dye + H_2O (peroxidase)

A 11 μl sample of each sonicate was used, and the reactions allowed to proceed for 5 min at 37°C prior to an absorbance measurement at 670 nm. The absorbance reading was read off on a standard curve, and the activity of CK determined by the analyzer.

f) Effect of activating the second messenger systems on thyrocyte morphology

Human thyrocytes were prepared and washed as described above. To wells in triplicate various additions were made such that 3 groups were created :- 1) TSH -, PMA/A23187 - ; 2) TSH +, PMA/A23187 -; and 3) TSH +, PMA/A23187 + (+/- denotes the presence or absence of TSH (1 U/L) or PMA/A23187 (both at 10^{-6}M). After an incubation period of 4

days the medium was removed, the thyrocytes were washed twice with 1 ml EBS and then fixed. Subsequently the fixed thyrocytes were photographed using a phase-contrast light microscope (x 20 magnification). The fixing and photography of the thyrocytes was kindly performed by Dr. D. Harrison of the Department of Pathology, University of Edinburgh.

g) Comparison of thyroidal ID-I activity in thyroid tissue from various thyroid disease states

Threetypesof human thyroid tissue (normal from around a carcinoma, multinodular goitre & Graves') were obtained from patients undergoing thyroid surgery and stored at -70°C. The different tissues were then homogenised in ID-I assay buffer to produce a 20% (w/v) homogenate, subjected to centrifugation (300g, 15 min) and then assayed for ID-I activity as described in Section 2.05a.

h) Differences in the affinity labelling of thyroid tissue homogenates from various thyroid disease states

Human thyroids were also homogenised in labelling buffer, diluted to a protein concentration of 1 g/L in labelling buffer, and affinity labelled as described in Section 2.08 in the presence or absence of PTU (500 μM) and rT_3 (10 μM).

4.02 : Results

a) Species differences in hepatic and thyroidal ID-I activity

There was a large variation in the activity of ID-I measured in the liver from the different species, but all showed substantial levels of activity ranging from 119 to 1045 fmol/min per mg protein (table 4a). However, only in the thyroid tissue from rat, man, mouse and guinea-pig could significant thyroidal ID-I activity be measured (1295, 283, 79 & 56 fmol

iodine released/min per mg protein respectively). All other species had very low thyroidal ID-I specific activities (0.3 - 3.6 fmol/min per mg, i.e. less than 0.3% of that of the rat).

b) Species differences in the affinity labelling of thyroidal homogenates

The liver homogenates from all species showed an ID-I band in the region of 27.3 to 29.0 kDa, though there were small but clear differences in the molecular weight of this band between species (table 4b). The identity of ID-I in the livers of all species was confirmed by the inhibition of affinity labelling by PTU/rT_3. A second band was also apparent in the liver of all species studied, which had a consistent molecular weight of 53.2 kDa. When thyroid homogenates were run on SDS/PAGE and autoradiographed, only rat and man exhibited a band corresponding in molecular weight to that of ID-I in the liver of the corresponding species (figure 4a; table 4b). The thyroids of the other species studied (sheep, llama, cattle, deer, pig and goat), exhibited bands within the molecular weight region of 30.5 - 33 kDa, though no band was found with the lower molecular weight corresponding to that of ID-I in the liver of the same species. The nature and identity of these other bands is unknown.

The presence or absence of the ID-I band in the thyroid of the various species correlated well with ID-I activity, thus indicating that the very low enzyme activities were due to a lack of expression of the enzyme, rather than an inhibition of activity. All the thyroid glands from these species had significant levels of glutathione peroxidase activity, often used as a marker of selenium status, thereby confirming that the lack of ID-I activity was not due to selenium deficiency (personal communication from Dr. J.R. Arthur).

c) Effect of TSH on thyroidal ID-I activity in human thyrocytes

Addition of TSH at concentrations of 10, 50, 100 and 200 mU/L to the thyrocytes had no significant effect on the level of ID-I activity measured in the thyrocyte sonicates after

culture (figure 4b). At TSH doses of 500 mU/L and above, a significant stimulation of ID-I activity was produced, with an apparent maximum stimulation of approximately 9-fold observed at 1 U/L. At higher doses of TSH the level of ID-I activity decreased significantly when compared to the optimum dose of 1 U/L TSH.

d) Effect on thyroidal ID-I activity of activating the different second messenger systems found in human thyrocytes

Once again TSH caused a significant stimulation of ID-I activity, (a 3-fold stimulation in the experiment illustrated, figures 4c). In the absence of TSH, the addition of PMA, A23187 or DMSO caused no significant change in the level of ID-I activity measured in the human thyrocytes. In contrast the addition of 8-bromo-cAMP resulted in a 3-fold increase in ID-I activity, similar in magnitude to that found with TSH at 1 U/L. When PMA and A23187 were added together in the absence of TSH a small decrease (≈ 33%) in ID-I activity was observed (figure 4c).

In the presence of TSH, a slight but not significant decrease in the level of ID-I activity was observed when PMA or DMSO were added (figure 4c). The addition of A23187 caused a 30% decrease, while 8-bromo-cAMP caused no further increase in ID-I above that observed with TSH alone. When added together, PMA and A23187 caused approximately a 4-fold decrease in ID-I activity when compared to the TSH control.

e) Changes in the affinity labelling of human thyrocytes after activation of the different second messenger systems

In the absence of TSH and PMA/A23187 the affinity label bound to a band with a molecular weight of 28.1 kDa, the same molecular weight as ID-I (figure 4d). When TSH only was present the intensity of the 28.1 kDa band was enhanced, signifying induction of the protein (ID-I) by TSH. In the presence of PMA and A23187 the intensity of the ID-I

affinity labelled band (M.W. 28.1 kDa) decreased significantly, wether TSH was present or not. Track 7 shows the labelling of human liver microsomes and once again the presence of ID-I, as well as 5 other bands (approximate M.W. of 32.0, 42.0, 43.0, 58.0 & 59.2).

PMA and A23187 in addition to reducing ID-I expression, also changed the expression of several other affinity labelled proteins. The intensities of five bands (M.W. ≈ 42.0, 44.5, 45.0, 58.0 & ≈ 59.0 kDa) were decreased with PMA/A23187, while the intensities of two other bands (≈ 24.0 and ≈ 25.8 kDa) were increased. The identity and function of most of these proteins is unknown, though the two 45 kDa proteins are of the same molecular weight as two mitochondrial isoenzymes of creatine kinase. In support of this the activity of creatine kinase in the cell sonicates was also found to decrease with the addition of PMA/A23187 (table 4c), while in the presence of TSH the activity of creatine kinase rose, and was accompanied by an increase in the intensity of the 45 kDa affinity labelled bands. The two proteins whose labelling was increased by PMA/A23187 (≈ 24.0 & 25.8 kDa) have approximately the same molecular weight as glutathione S-transferases (GSTs), a group of proteins known to be involved in drug metabolism. One could therefore speculate as to the identity of these labelled bands, though further investigation would be required to confirm their identity.

In addition to the changes in protein expression seen with the affinity label, other changes were observed on visual inspection of the gel when stained with Coomassie blue (figure 4e). The intensity of six bands decreased with PMA/A23187 (tracks 2,4 & 6) in the presence (tracks 3 - 6) or absence (tracks 1 & 2) of TSH, M.W. ≈ 27.5, 35.0, 36.0, 37.5, 44.5 and 45.0 kDa. Four other proteins with M.W. ≈ 26.5, 26.75, 33.5 & 34.0 kDa were apparently induced by PMA/A23187. The identity of these proteins is unknown at present.

f) Effect of activating the second messenger systems on thyrocyte morphology

Figure 4f shows the thyrocytes from all 3 groups grown in monolayer culture. In the absence of TSH no follicles were observed, while the addition of TSH (1 U/L) caused the formation of follicles. When PMA and A23187 were present in addition to TSH, no follicles were observed.

g) Comparison of thyroidal ID-I activity in thyroid tissue from various thyroid disease states

The level of ID-I activity measured in normal and multinodular thyroid tissues were statistically indistinguishable and ranged between 8 - 46 fmol/min per mg protein (figure 4g). In contrast, the ID-I level in tissues from patients with Graves' disease were significantly higher, with a range of activities from 116 - 246 fmol/min per mg protein.

h) Differences in the affinity labelling of thyroid tissue homogenates from various thyroid disease states

Affinity labelling of multinodular goitre, Graves' disease and normal tissue resulted in the appearance of approximately 8 predominant radiolabelled bands, including ID-I at a molecular weight of 28.1 kDa (figure 4h). The presence of PTU and rT_3 during labelling resulted in the disappearance of the 28.1 kDa band, once again providing strong evidence that the 28.1 kDa band is ID-I. Normal and multinodular tissues generally showed low to moderate ID-I band intensity, with higher ID-I band intensity generally seen in the Graves' disease tissues.

Table 4a : Activity of ID-I in the thyroids and livers of the various species listed, expressed as the amount of iodine released from rT_3/min per mg protein.

Species	**Thyroidal Activity** (fmol/min/mg)	**Hepatic Activity** (fmol/min/mg)
Rat	1296	1045
Guinea-Pig	283	-
Mouse	79	-
Human	56	437
Goat	3.6	1052
Cattle	1.9	1066
Rabbit	0.9	-
Sheep	0.4	170
Pig	0.4	291
Llama	0.3	119
Deer	ND	-

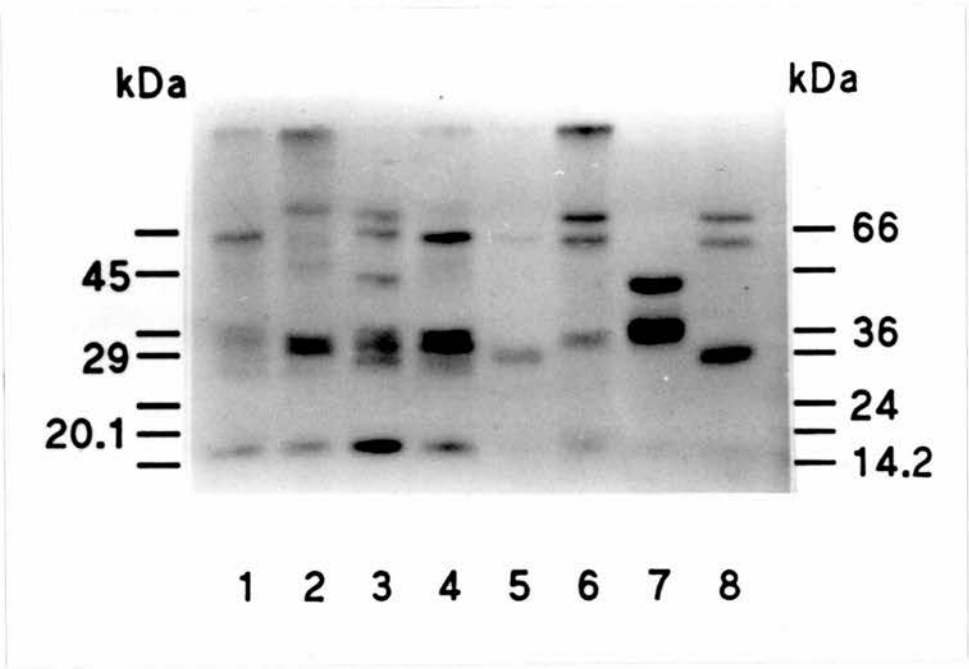


Figure 4a : Autoradiography of an SDS-PAGE gel of thyroid homogenates from various species labelled with bromoacetyl-[^{125}I]-rT_3. Tracks 1-8 show sheep, llama, human, cattle, deer, pig, goat and rat respectively. The mobilities of molecular mass markers are also indicated.

Table 4b : Molecular weight of hepatic ID-I from various species as determined by autoradiography of an SDS-PAGE gel of homogenates labelled with bromoacetyl-[^{125}I]-rT_3 affinity label in the presence and absence of PTU and rT_3 (inhibitors of ID-I labelling). Affinity labelled bands observed within the molecular mass region of hepatic ID-I in the corresponding thyroid homogenates are also shown. Only human and rat thyroid tissue expressed a protein band with the corresponding molecular weight of hepatic ID-I in the same species (denoted by *). This data was produced by Dr. J.R.Arthur and F. Nicol of the Rowett Research Institute, Aberdeen, U.K as part of a collaborative study.

Species	**Molecular mass of affinity labelled protein (kDa)**	
	Liver (ID-I)	**Thyroid**
Rat	28.1	28.1*
Human	28.1	28.1*
Goat	27.3	32.9
Cattle	28.9	32.9
Sheep	27.3	30.4
Pig	28.1	31.6
Llama	28.1	30.5

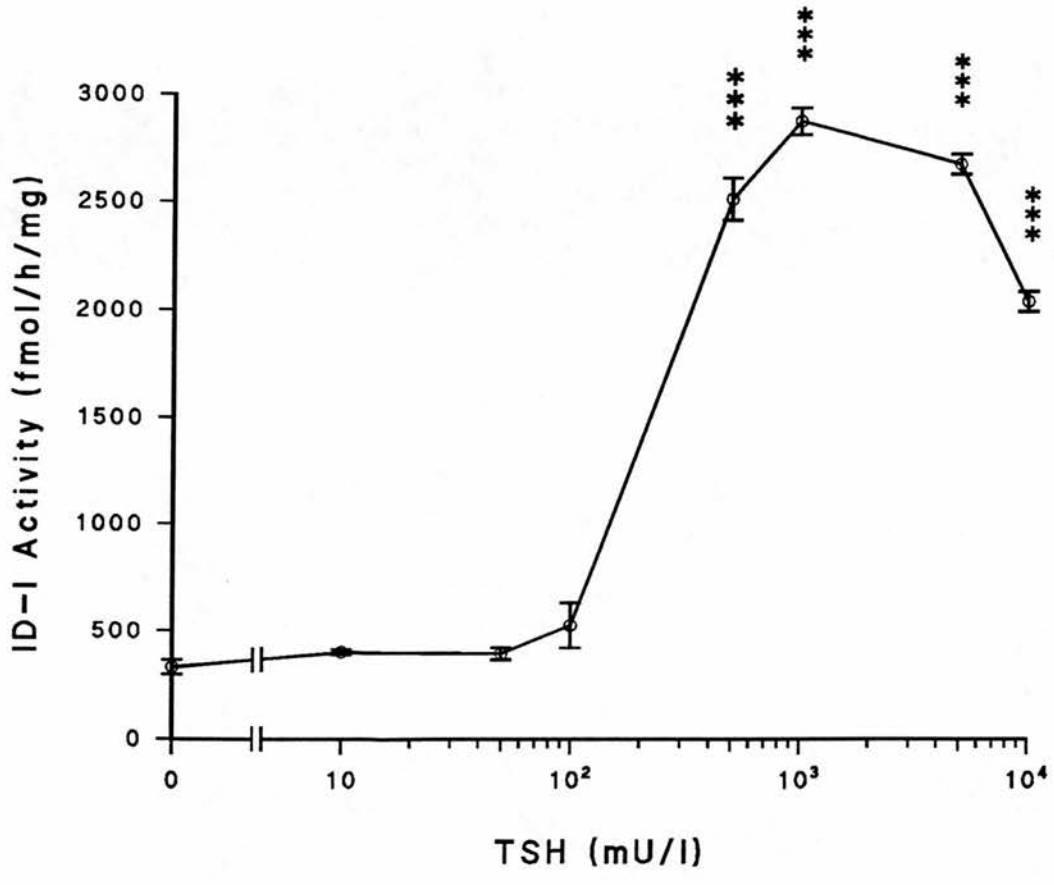
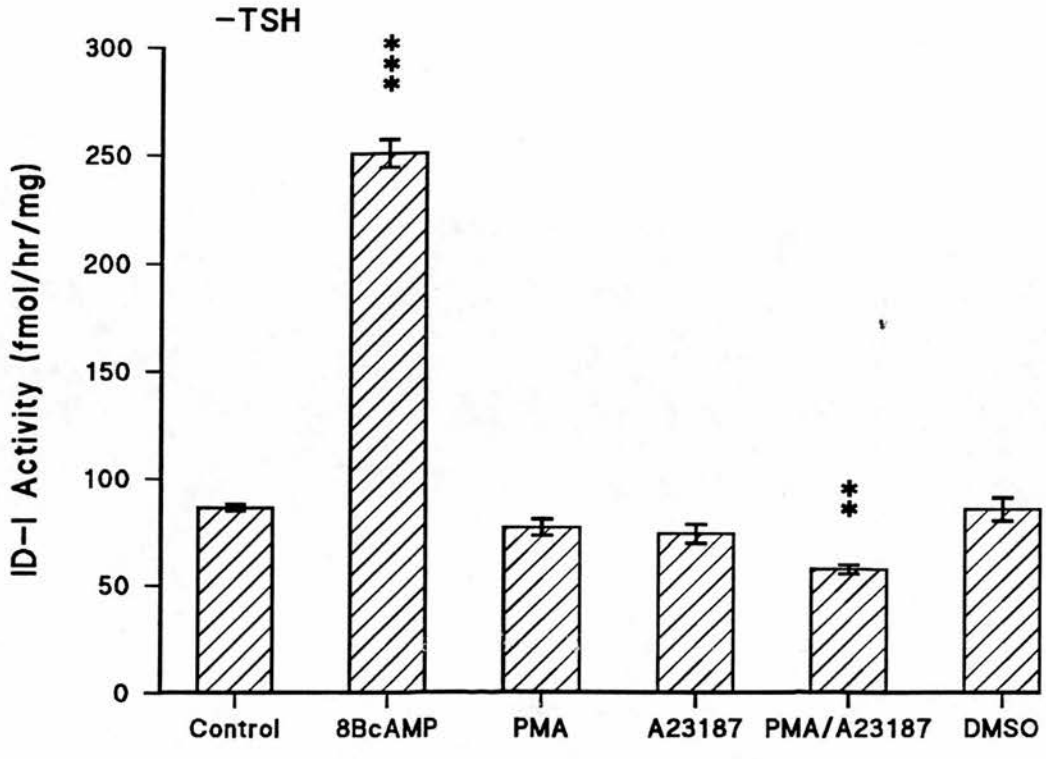


Figure 4b : Thyrocytes were cultured in the presence of 100 nmol/L sodium selenite and concentrations of TSH from 0 to 10 U/L. Values shown are mean ± SEM of triplicate incubations in a representative experiment carried out on at least three occasions. Significant differences in ID-I activity from the control (- TSH) were observed at TSH concentrations above 500 mU/L. Concentrations of TSH in excess of 5 U/L caused a significant decrease in ID-I activity compared to TSH at 1 U/L ($p < 0.05$).

Figure 4c : Thyrocytes were cultured in the presence of 100 nmol/L sodium selenite, in the presence or absence of TSH, with further additions to wells in triplicate of 8-Bromo-cAMP (10^{-4}M), PMA (10^{-6}M), A23187 (10^{-6}M), PMA with A23187 (10^{-6}M) and DMSO (1% v/v). Values shown are mean ± SEM in a representative experiment carried out on at least 3 occasions. Significant differences in ID-I activity from the control values were observed with 8-Bromo-cAMP and PMA/A23187 when in the absence of TSH, and with A23187 and PMA/A23187 in the presence of TSH.


-TSH
ID-I Activity (fmol/hr/mg)
300
250
200
150
100
50
0
***
**
Control
8BcAMP
PMA
A23187
PMA/A23187
DMSO

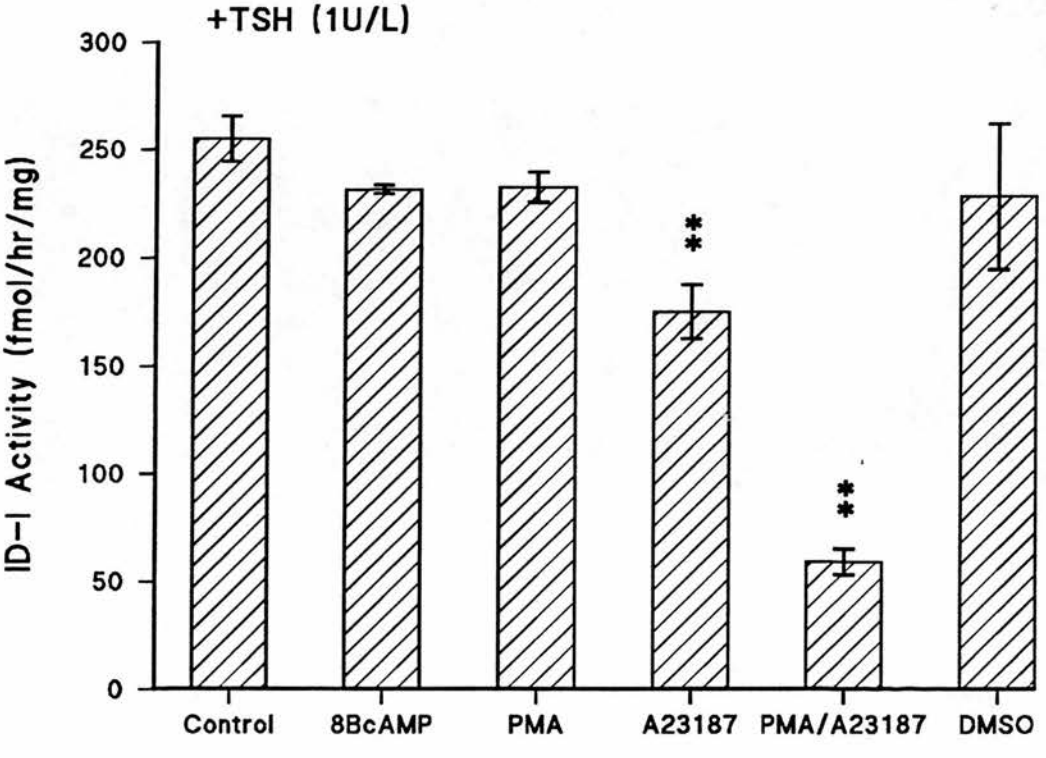

+TSH (1U/L)
ID-I Activity (fmol/hr/mg)
300
250
200
150
100
50
0
**
**
Control
8BcAMP
PMA
A23187
PMA/A23187
DMSO

Figure 4d : Autoradiography of an SDS-PAGE gel of thyrocyte sonicates labelled with bromoacetyl-[^{125}I]-rT_3 affinity label after growth in the presence or absence of various compounds. Tracks 1 & 2 show affinity labelling of thyrocytes in the presence of TSH, with (track 1) and without (track 2) PMA/A23187 present. Tracks 3 - 6 show affinity labelling of thyrocytes grown in the presence (tracks 3 & 4) or absence (tracks 5 & 6) of TSH, with (track 3 & 5) and without (track 4 & 6) PMA/A23187 present. Track 7 shows human liver microsomes labelled with the affinity label as a control. The mobilities of the molecular mass markers and ID-I are also indicated.

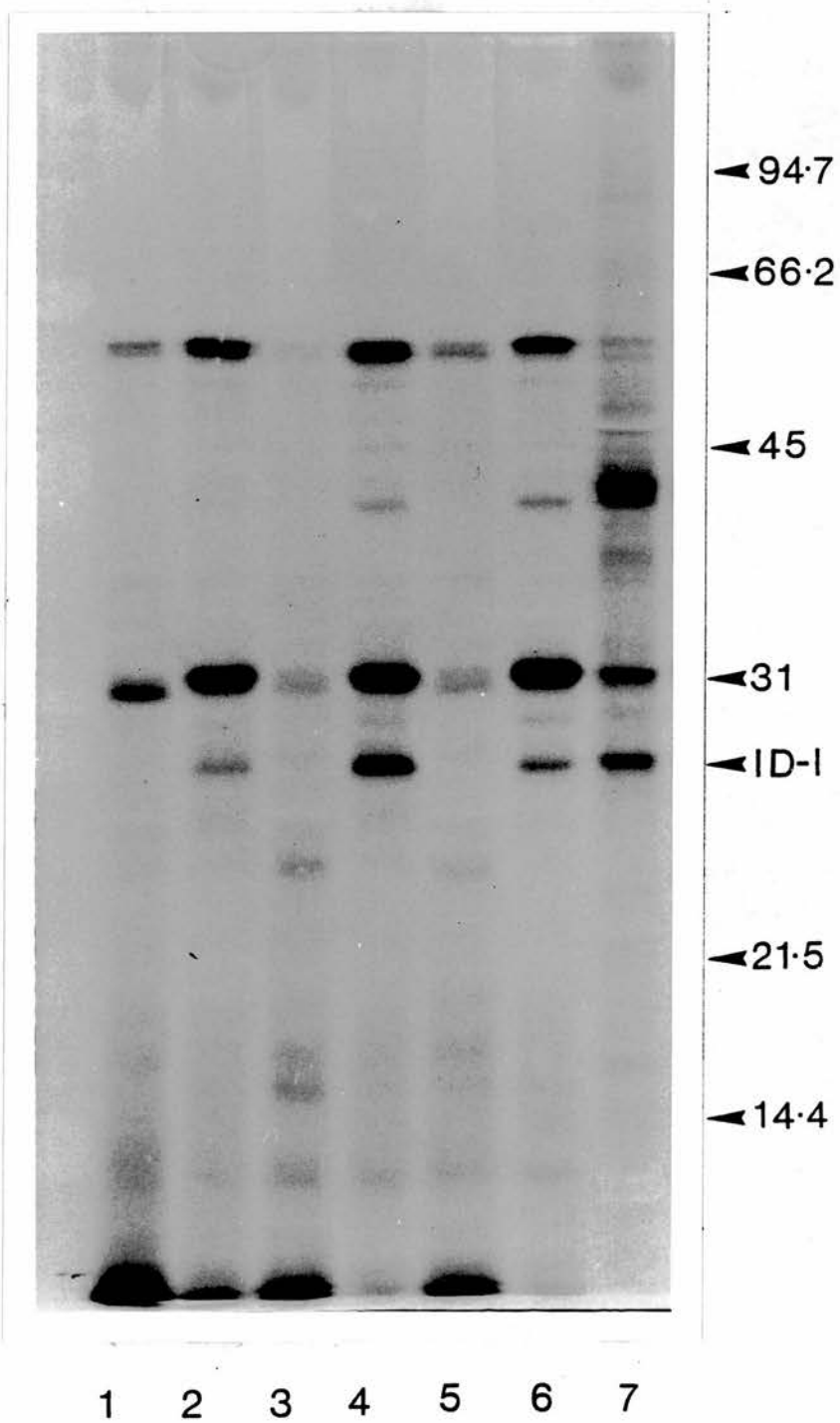

94·7
66·2
45
31
ID-I
21·5
14·4
1 2 3 4 5 6 7

Table 4c : Creatine kinase activity measured in human thyrocytes grown in the presence or absence of TSH and in the presence or absence of PMA and A23187. This data was produced with the assistance of Dr. A.F. Howie.

Thyrocyte Growth Conditions	Creatine Kinase Activity
- TSH, - PMA/A23187	0.274 U/mg protein
- TSH, + PMA/A23187	Not Dectectable
+ TSH, - PMA/A23187	0.622 U/mg protein
+ TSH, + PMA/A23187	Not Dectectable

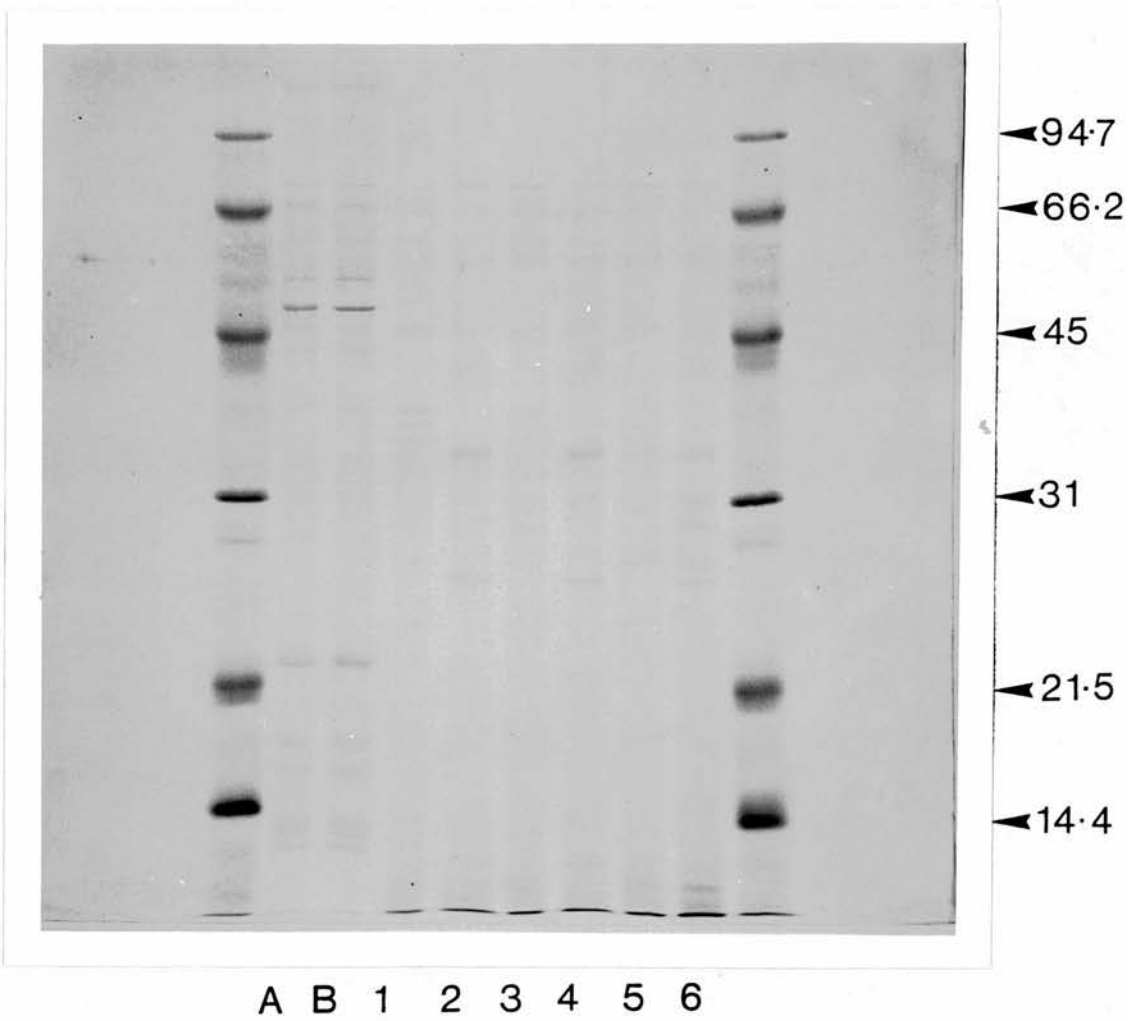
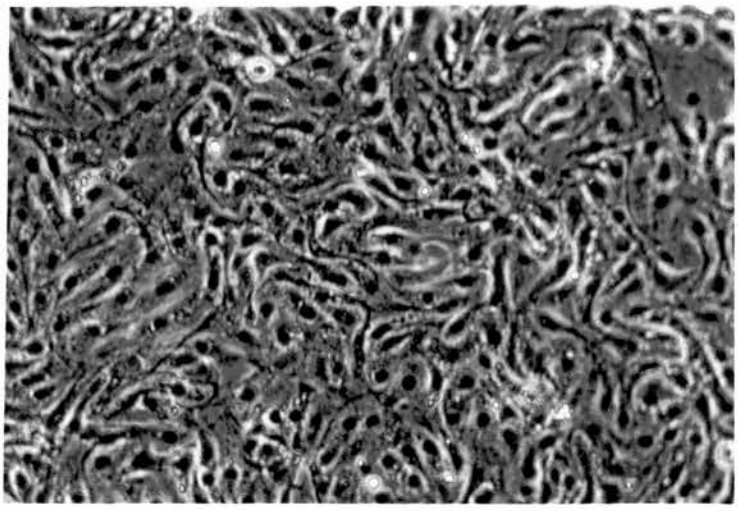


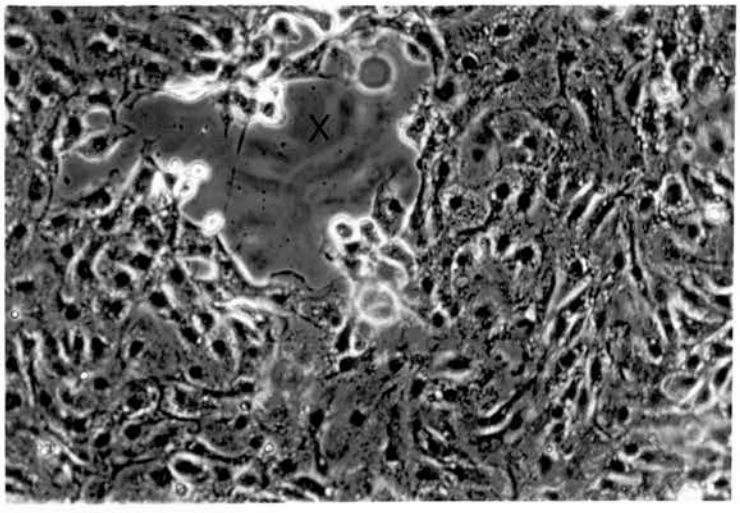
Figure 4e : Photograph of a Coomassie-blue stained SDS-PAGE gel of thyrocyte sonicates after growth in the presence or absence of various compounds. Tracks 1 - 4 show thyrocytes grown in the presence (tracks 3 & 4) or absence (tracks 1 & 2) of TSH, with (tracks 2 & 4) and without (track 1 & 3) PMA/A23187 present. Tracks 5 and 6 show thyrocytes from a different preparation grown in the presence of TSH, with (track 6) and without (track 5) PMA/A23187. Tracks A and B show human liver microsomes. The mobilities of the molecular mass markers are also indicated.

Figure 4f : Photographs of human thyrocytes grown in the presence of various compounds (Photograph A (no additions); Photograph B (TSH +, PMA/A23187 -); and Photograph C (TSH +, PMA/A23187 +). The follicle consisiting of colloid overlying the monolayer in photograph B is denoted by X.

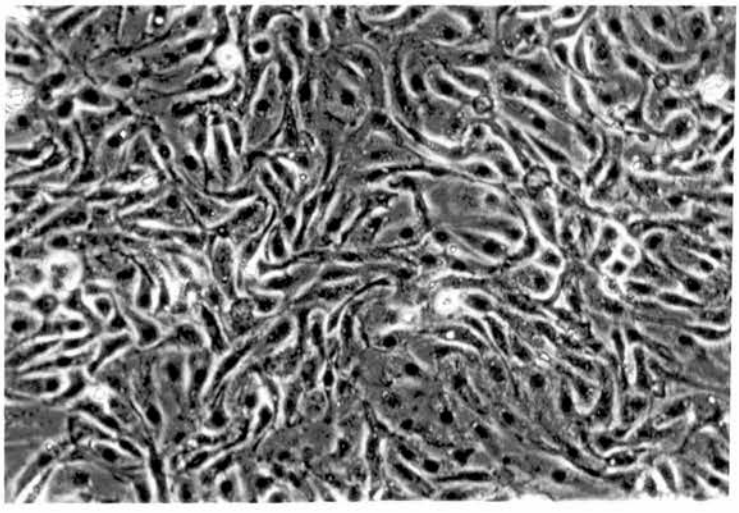
A



B



C



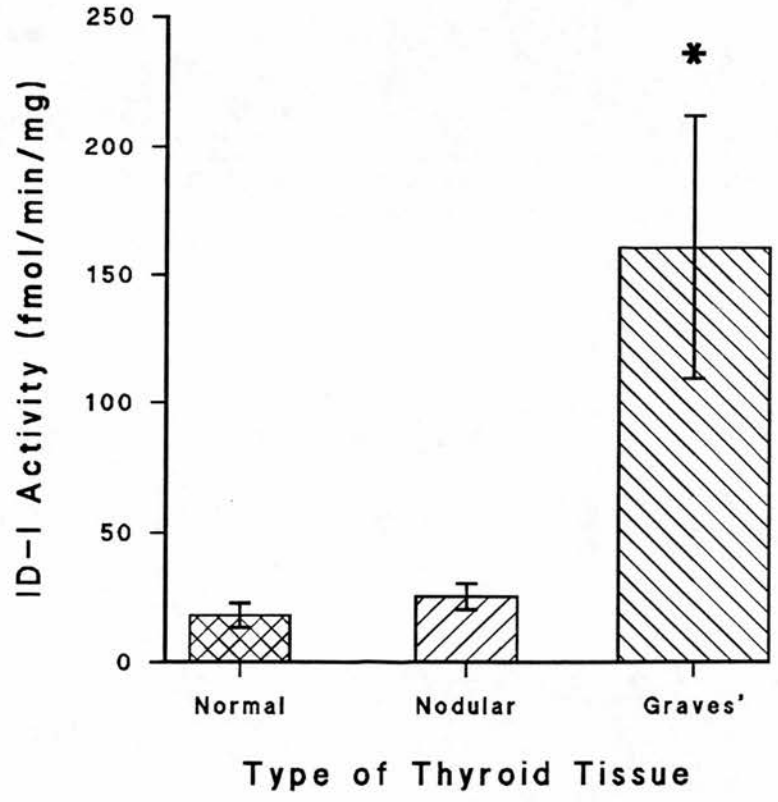
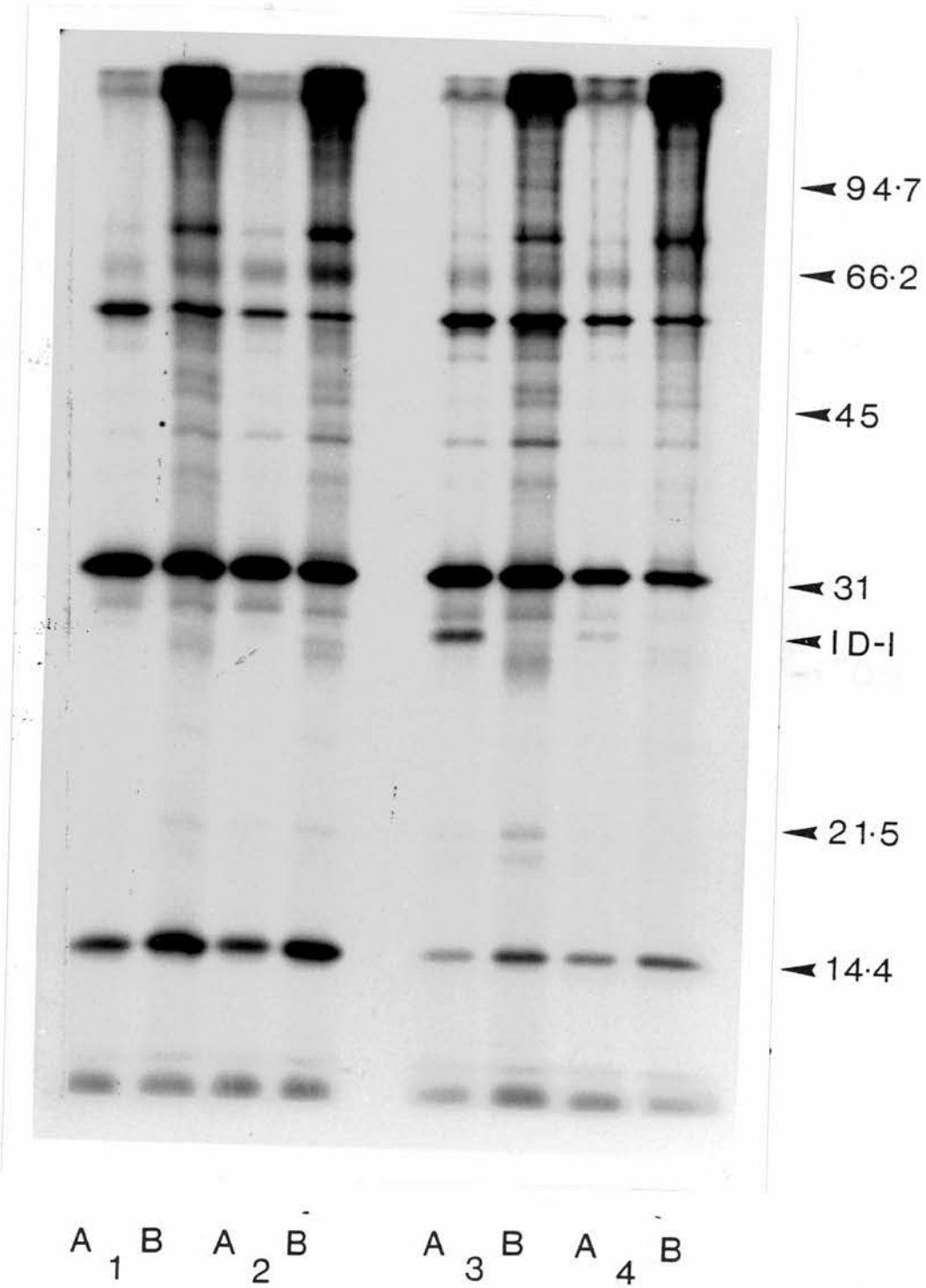
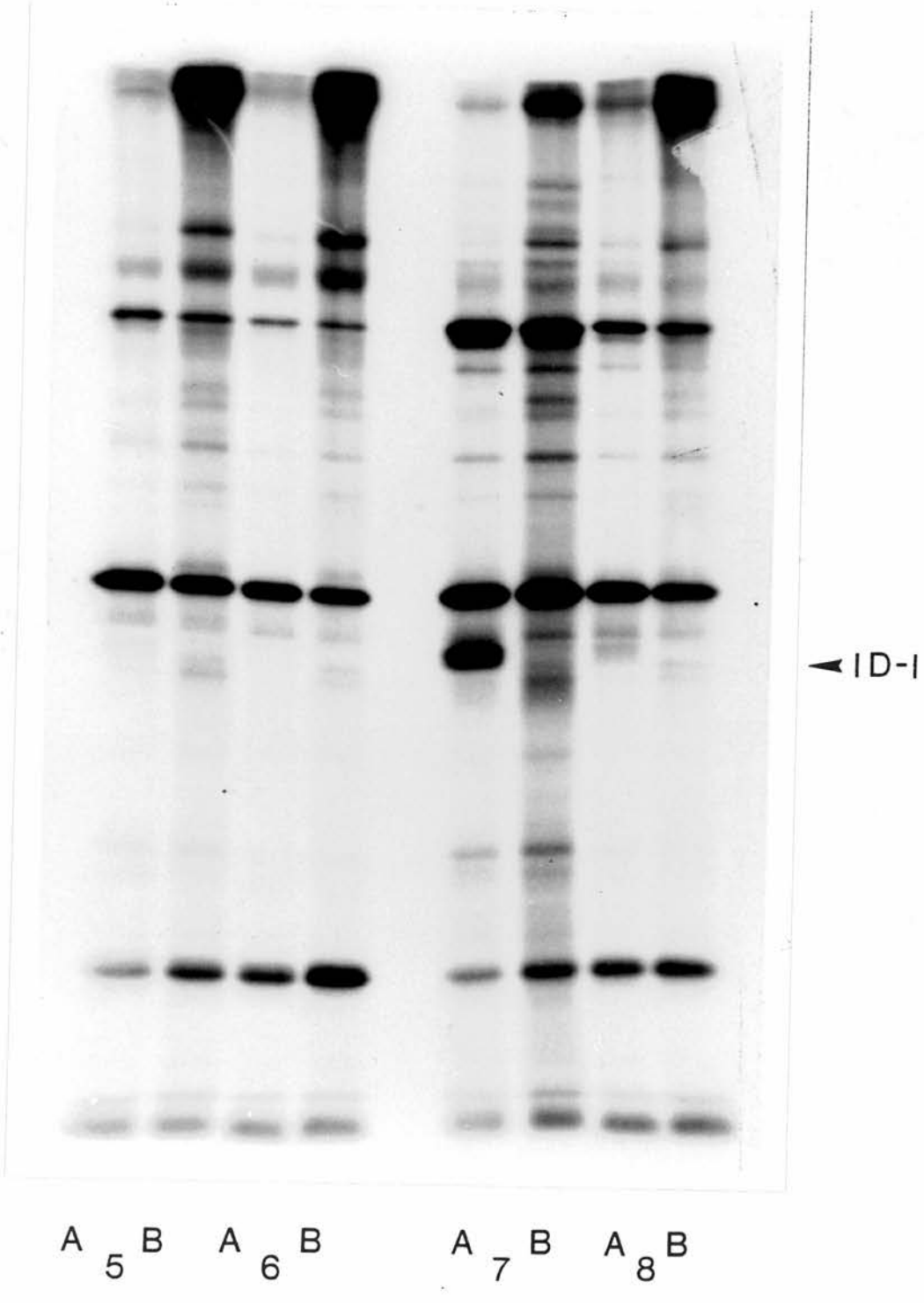
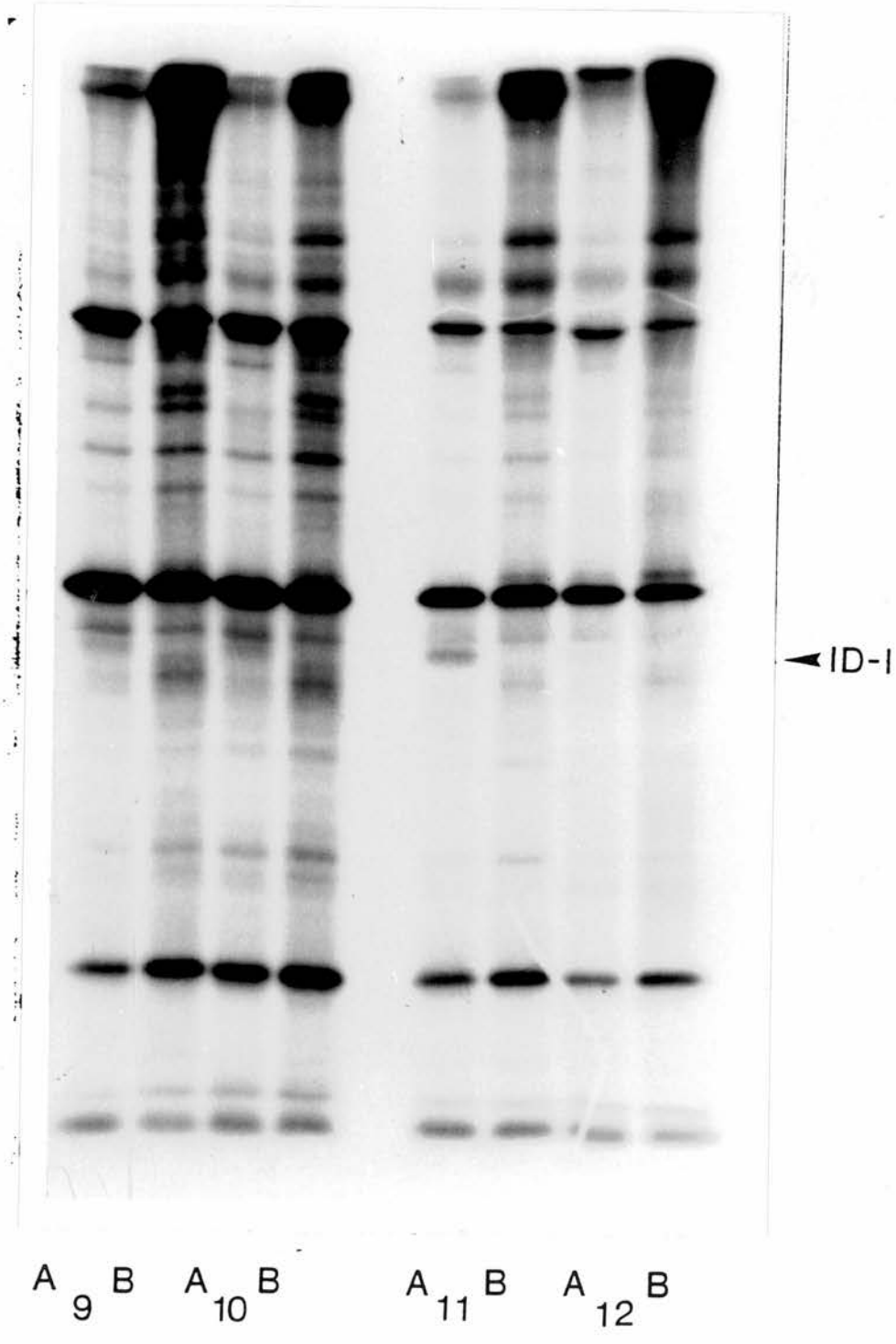


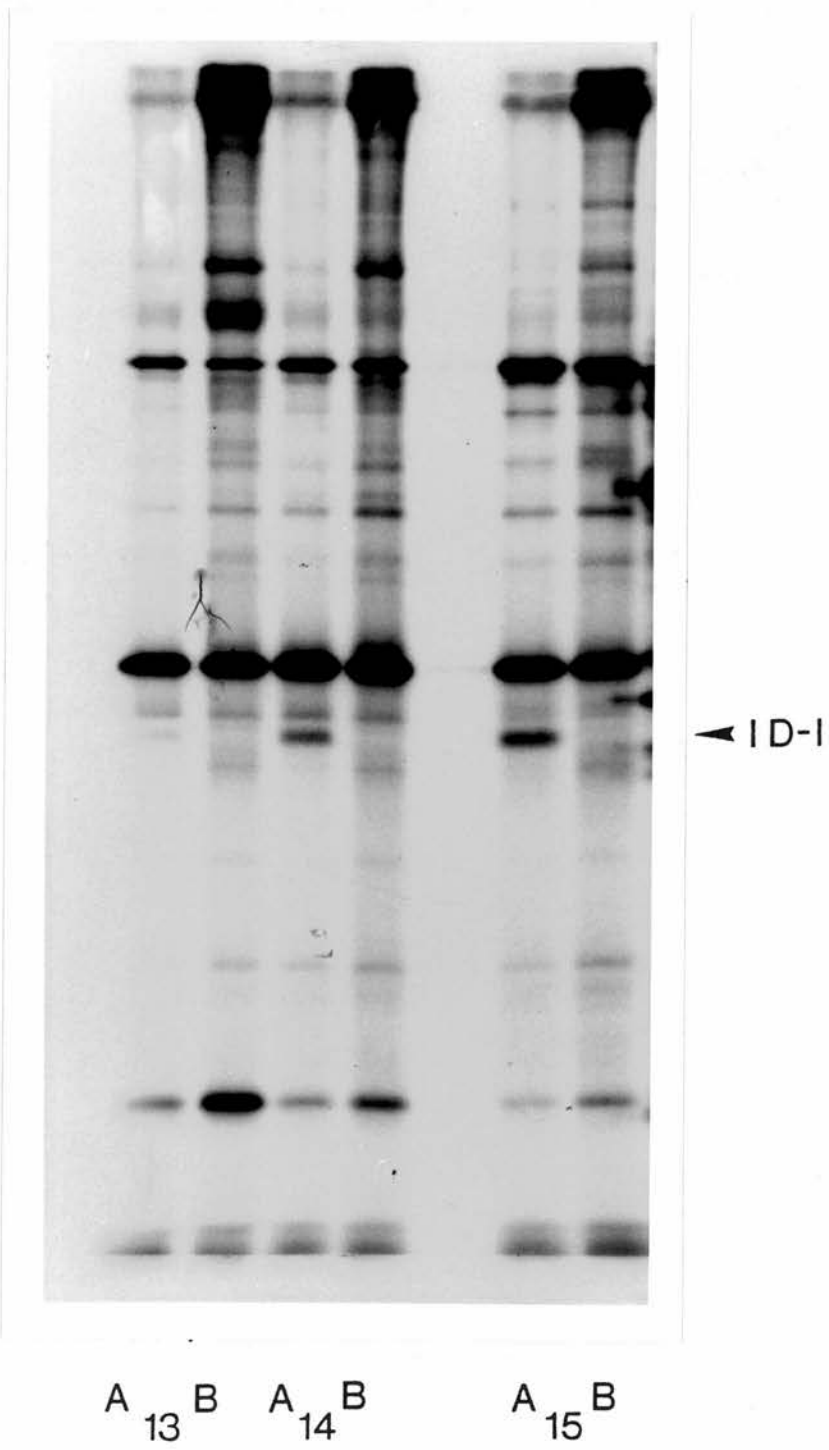
Figure 4g : Comparison of the level of ID-I activity found in normal, multinodular and Graves' tissue as determined by the release of ^{125}I from [^{125}I]-rT_3. No significant difference in ID-I activity was demonstrated between normal and nodular tissue, while ID-I activity in Graves' tissue was significantly higher than either normal or nodular hyperthyroid tissue (5 patients per group, $p < 0.05$ using a Mann-Whitney unpaired t-test).

Figures 4h : Autoradiography of SDS-PAGE gels of thyroid homomgenates from three types of thyroid tissue labelled with bromoacetyl-[^{125}I]-rT_3 in the absence (a) or presence (b) of rT_3/PTU. Tracks 2, 4 & 8 show normal tissue, tracks 3, 7, 11, 14 & 15 show Graves', with the remaining tracks (1, 5, 6, 9, 10, 12, 13) showing multinodular tissue. The mobilities of the molecular mass markers and ID-I are also indicated.


94·7
66·2
45
31
ID-I
21·5
14·4
A 1 B
A 2 B
A 3 B
A 4 B


ID-I
A 5 B
A 6 B
A 7 B
A 8 B


ID-I
A 9 B
A 10 B
A 11 B
A 12 B


ID-I
A 13 B
A 14 B
A 15 B

4.03 : Discussion

All of the species studied showed significant hepatic ID-I expression (table 4a), but with slight variations in the molecular weight of this enzyme (table 4b). These findings confirm those of other workers (Schoenmakers *et al,* 1992) who have shown that, in general all species have hepatic ID-I with similar turnover rate and specific activity, but with variation in molecular weight between 25.7 and 29.1 kDa. However, Schoenmarkers *et al* also found that the dog, for reasons not explained, exhibited much lower rates of ID-I turnover than the other species studied (man, mouse, rabbit, cow, pig, sheep, goat, chicken or duck), and like the rat, the dog had much higher levels of hepatic ID-I. In contrast, the expression of thyroidal ID-I shows a much higher degree of species variation (table 4a). As previously reported the human thyroid expresses ID-I (Ishii *et al,* 1981; Toyoda *et al,* 1992) and in addition, the thyroids of rat, guinea-pig and mouse also contained significant levels of ID-I activity. The thyroids of the other species studied (sheep, llama, cattle, deer, pig, goat and rabbit) showed barely detectable ID-I activity (< 0.3% of the activity of that found in the rat) (table 4a).

Affinity labelling of the thyroid homogenates demonstrated variation in the intensity of the ID-I band, as determined by autoradiography (figure 4a), which paralleled the activity measurements. Thus, the variation in thyroidal ID-I activity between the species is as a result of differences in the level of ID-I expression, and not differences in the specific activity of the enzyme. To generalise, it would also appear that herbivores and the pig lack thyroidal ID-I, while omnivores (rat, mouse, guinea-pig and man) express significant levels of thyroidal ID-I. Why demarcation exists is unclear, though it may be relevant that herbivores lack a potential source of plasma T_3, which may become important in conditions where TSH is elevated, such as in iodine deficiency.

In agreement with other workers I have found human thyroidal ID-I to be responsive to TSH (figure 4b), and that the stimulation of ID-I expression is mediated via

the cAMP second messenger pathway (figure 4c) (Ishii *et al,* 1983). The level of ID-I activity was increased in a dose-dependent manner when TSH was added, with a maximum response seen at 1 U/L TSH. Above this dose a significant decrease in activity occurred. Previous workers have shown that high TSH concentrations stimulate the Ca^{2+}-phosphatidylinositol (Ca^{2+}-PI) cascade in human thyroid slices and human thyrocytes in primary culture (Laurent *et al,* 1987; Laurent *et al,* 1989; Raspe *et al,* 1991; Maenhaut *et al,* 1990). These workers demonstrated increases in the concentrations of inositol-1,4,5 phosphate (IP_3) and intracellular calcium (Ca^{2+}_i) when 10 U/L TSH was added. They concluded that TSH, at concentrations 10-fold higher than those required for maximal stimulation of the cAMP cascade, activates phospholipase C, which catalyses the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) to yield IP_3 and diacylglycerol (DAG). The ability of TSH to stimulate both the cAMP and Ca^{2+}-PI cascades has been reported to occur through two distinct types of TSH receptor (Lefort *et al,* 1984; Laurent *et al,* 1987). In this regard, several other hormones and neurotransmitters (e.g. vasopressin and glucagon) have been shown to stimulate both pathways through different receptors (Jard, 1983; Wakelam and Murphy, 1986). However, more recent work using Chinese hamster ovary (CHO) cells transfected with the cDNA for the human TSH receptor has shown that both pathways can be stimulated by TSH via one type of TSH receptor, possibly by activation of G_s and G_q transducing GTP binding proteins (Van Sande *et al,* 1990).

The decrease in ID-I activity I found on addition of high concentrations of TSH (figure 4b, TSH > 5 U/L) could be explained by activation of the Ca^{2+}-PI pathway. This hypothesis is supported by the data in figure 4c, where the phorbol ester, PMA and the calcium ionophore, A23187 were added together in the presence of TSH (1 U/L). As a result, a marked decrease in ID-I activity to basal levels was observed. Addition of PMA and A23187 together is known to simulate the combined effects of DAG and Ins 1,4,5 P_3

respectively. It is logical therefore that stimulation of the Ca^{2+}-PI pathway by other agents might also result in a reduction in ID-I activity. Accordingly, ATP, bradykinnin and thyrotropin-releasing hormone (TRH) might also modify ID-I activity as these agents have been shown to stimulate the Ca^{2+}-PI pathway in primary cultured human thyrocytes (Raspe *et al,* 1991). Stimulation of the Ca^{2+}-PI pathway by ATP has also been shown to occur in the rat thyroid cell line FRTL-5, possibly through a P_2 purinergic receptor (Okajima *et al,* 1988). In conjunction with this stimulation of the Ca^{2+}-PI cascade, ATP can inhibit cAMP accumulation, probably by inhibition of adenylate cyclase rather than activation of phosphodiesterase, the enzyme responsible for hydrolysis of cAMP (Okajima *et al,* 1989). These workers proposed that the inhibition of adenylate cyclase by ATP was mediated by activation of the P_{2i} or the A_1 purinergic receptor subtype, with these receptors making use of a G_i or G_i-like transducer protein. It is noteworthy that ATP is often co-released from cholinergic and adrenergic nerves (Gordon, 1986; Burnstock and Kennedy, 1986), and that the thyroid has been shown to have extensive cholinergic and adrenergic innervation (Melander, 1986). In addition, TRH and bradykinnin have also been shown to act as neurotransmitters (Emerson, 1986; Steranka *et al,* 1988). It is interesting therefore to speculate that release of ATP, TRH or brabykinnin could occur in the thyroid, thereby giving rise to decreased ID-I expression via activation of the Ca^{2+}-PI cascade (Raspe and Dumont, 1992). In fact ATP could reduce ID-I expression both by the stimulation of the Ca^{2+}-PI cascade and by the inhibition of the cAMP cascade.

The level of ID-I expression in the thyrocytes was determined in the presence and absence of various compounds (TSH, PMA/A23187) by comparing the intensity of the affinity labelled proteins visualized by autoradiography (figure 4d). The level of ID-I activity was found to correlate closely with the level of ID-I expression, as determined from the intensity of the affinity labelled ID-I band. TSH (1 U/L) caused an increase in the level of ID-I expression, while PMA/A23187 (both at 10^{-6}M) caused a clear decrease in expression

of the ID-I band (M.W ≈ 28.1 kDa). This data confirms that activation of the Ca^{2+}-PI pathway does cause reduced ID-I expression.

In addition to labelling ID-I, the affinity label also bound to several other proteins, both in the human liver microsomes and in the human thyrocyte sonicates (figure 4d). Five major bands were seen in the human liver microsomes (M.W. ≈ 32.0, 42.0, 43.0, 58.0 & 59.2 kDa). The identity of these proteins remains to be investigated. Human thyrocytes exhibited several proteins whose expression changed on addition of PMA/A23187. The expression of three unidentified proteins was inhibited by PMA/A23187 (M.W. ≈ 42.0, 58.0 & 59.2), as was the expression of two proteins with molecular weights of 44.5 and 45.0 kDa. Kohrle *et al* identified two isoenzymes of mitochondrial creatine kinase (Mi-CK) with molecular weight of approximately 45 kDa, using the affinity label bromoacetyl-[^{125}I]-T_3 (Wyss *et al,* 1993). In conjunction with the observed decrease in intensity of the two 45 kDa affinity labelled bands, I have also shown a decrease in the creatine kinase (CK) activity when PMA/A23187 was present. TSH appeared to stimulate CK activity and the expression of the two ≈ 45 kDa proteins when PMA/A23187 was absent (table 4c, figure 4e). It is possible therefore that the two 45 kDa proteins are mitochondrial isoenzymes of CK, although further investigation is required to confirm the identity of these proteins, possibly via the use of Western blotting or mRNA identification techniques.

The affinity labelled band intensities of two proteins (M.W. ≈ 24.0 & 25.8 kDa) were increased by PMA/A23187. These proteins are approximately the same molecular weight as the subunits of the glutathione S-transferases (GST) iosenzyme family. GSTs are a family of multigene, cytosolic, phase 2 detoxification enzymes of which there are at least seven in man. This group of isoenzymes catalyses the conjugation of reduced glutathione to numerous electrophilic, hydrophobic compounds in the first step of mercapturic acid formation. Certain GST isoenzymes have been demonstrated to have peroxidase activity and are able to reduce organic hydroperoxides to their respective alcohols. Potential

substrates for GSTs include fatty acids and DNA, and hence GST peroxidase activity may represent part of a repair mechanism for both DNA and lipids. Non-catalytic properties of GSTs include ligand-binding of various organic compounds such as bilirubin, steroids and bile salts (Beckett and Hayes, 1993). Once again further investigation is required to confirm the identity of these proteins.

Further to the changes seen in protein expression using the affinity label, PMA/A23187 caused the expression of several other proteins to change, as assessed when the gels were stained with Coomassie blue and visually inspected (figure 4e). Six proteins decreased with PMA/A23187 (M.W. ≈ 27.5, 35.0, 36.0, 37.5, 44.5 & 45.0 kDa), while four proteins were induced (M.W. ≈ 56.7, 26.75, 33.5 & 34.0). As described above the ≈ 45.0 kDa protein bands could be mitochondrial isoenzymes of creatine kinase. The other proteins are yet to be identified. It is clear therefore that PMA and A23187 have profound effects on the expression of a wide range of proteins, most probably refecting the ability of this combination to simulate the Ca^{2+}-PI cascade. In addition to the changes in protein expression observed with PMA/A23187, these compounds when added together caused inhibition of TSH-induced follicle formation (figure 4f). The observed changes in protein expression may also reflect changes in cell growth and the degree of thyrocyte differentiation.

When thyroidal ID-I activity was measured in the thyroid tissue from different thyroid disease states, a large degree of variation was observed. The level of ID-I activity measured in normal and multinodular hyperthyroid tissues was similar and ranged between 8 - 46 fmol/min/mg (figure 4g). It is not known if the normal regulatory mechanisms controlling ID-I expression operate in nodules where there is automonous synthesis of thyroid hormones. Certainly if the expression of ID-I is still under the control of TSH, one might expect that the ID-I activity should be similar in normal tissue and nodular tissue since preoperatively both types of patients would have been rendered

euthyroid (normal TSH levels) by pharmacological manipulation. The thyroid tissues taken from patients with Graves' disease had significantly higher levels of ID-I activity ranging from 116 - 246 fmol/min/mg (Mann-Whitney t-test, $p < 0.014$). Other workers also noted increased ID-I activity in the thyroid tissue from patients suffering from Graves' disease (Ishii *et al,* 1981). This increase is likely to be due to the presence and activity of thyroid stimulating immunoglobulins (TSIs). It has been shown that TSIs can stimulate the adenylate cyclase of human thyrocytes by binding to the TSH receptor (McKenzie and Zakarija, 1976; Van Sande *et al,* 1980; Field *et al,* 1980). Stimulation of adenylate cyclase via the TSH receptor is therefore most probably responsible for this increase in ID-I activity. However unlike TSH, excessive concentrations of TSIs for reasons unknown, are unable to stimulate the Ca^{2+}-PI cascade in human thyroid slices (Laurent *et al,* 1991). These workers therefore concluded that the pathogenesis of Graves' disease can be fully accounted for by the ability of TSIs to stimulate adenylate cyclase.

The thyroid tissues which exhibited elevated ID-I activity also exhibited higher ID-I band intensity when affinity-labelled with bromoacetyl-[^{125}I]-rT_3 (figure 4h). Labelling the thyroid homogenates in the presence of rT_3 (10 μM) and PTU (500 μM), compounds which bind to ID-I and interfere with the binding of the affinity label, resulted in the disappearance of the 28.1 kDa band, thereby confirming the identity of this band as ID-I. Therefore, those tissues with high ID-I activity demonstrate increased ID-I expression as recently described (Toyoda *et al,* 1992), rather than an increase in the specific activity of the enzyme. The ability of TSIs to stimulate ID-I expression, with no obvious mechanism to guard against excessive over expression, may therefore be an important factor in the pathogenesis of Graves' disease (Ishii *et al,* 1981; Toyoda *et al,* 1992). Indeed, during Graves' disease there is an increase in the proportion of T_3 produced directly by the thyroid (Larsen, 1975), and ID-I could well produce a significant amount of this thyroidal formed T_3.

Section 5 : THE SELENIUM-DEPENDENCE OF ID-I IN HUMAN THYROCYTES GROWN IN PRIMARY CULTURE

In addition to ID-I, various other selenoproteins have also been reported in the thyroid gland of rats (Behne *et al,* 1988). Behne *et al* showed that when [^{75}Se]-selenite was administered to rats, 11 proteins were labelled with ^{75}Se in the thyroid. These proteins had molecular weights of 12.1, 15.6, 18.0, 19.7, 23.7, 27.8, 55.5, 59.9, 64.9, 70.1 and 75.4 kDa. The protein with a molecular weight of 23.7 kDa is most probably a subunit of glutathione peroxidase (GPx) (Rotruck *et al,* 1973), the enzyme responsible for the protection of tissue from peroxidative damage. The protein with a molecular weight of 27.8 Kda was shown to be ID-I (Beckett *et al,* 1987; Behne *et al,* 1988; Behne *et al,* 1990; Kohrle *et al,* 1990a; Kohrle *et al,* 1990b). The function and role of the remaining proteins remains to be elucidated, however some of these proteins may well have important biological roles, since in selenium deficiency they appear to be supplied with selenium in preference to glutathione peroxidase (Behne *et al,* 1988). In selenium deficiency ID-I is also supplied with selenium in preference to GPx, since rat hepatic ID-I activity was decreased to 10% of the activity found in selenium-supplemented rats, while GPx decreased to less than 1% of the supplemented animals (Beckett *et al,* 1987; Beckett *et al,* 1989; Arthur *et al,* 1990d).

In addition to its roles in GPx and ID-I activity, selenium has also been shown to have various other important functions. *In vitro* experiments have shown selenium is required for cell growth, but inhibits the growth of cells in a reversible manner at high, but sub-toxic levels (Medina and Oborn, 1981; Medina and Oborn, 1984; Medina *et al,* 1985). Similar levels of selenium were also shown *in vivo* to act in an anti-carcinogenic manner in various animal models (Medina, 1986; Ip, 1986; Combs and Combs, 1986a). Clearly selenium has far reaching and widespread roles in biological systems.

In this section I have studied the effects of selenium supply on thyroidal ID-I using primary cultured human thyrocytes. I have thenproceededo investigate the effects of adding gold thioglucose (a compound that bind to selenocysteine) to human and sheep thyrocytes in primary culture. In addition I have investigated selenium trapping by human thyrocytes, and also studied the expression of various selenoproteins found in human thyrocytes.

5.01 : Methods

a) Effect of selenium on ID-I activity in human thyrocytes

Human thyrocytes were prepared as described in Section 2.03 from normal, multinodular or Graves' thyroid tissue. The thyrocytes were plated out in 12 well plates at a cell density of $5x10^5$ cells/well in 1 ml of medium (DMEM/10% CPSR-5) and incubated for 24 hours in a 37°C, 5% CO_2 incubator. After 24 h, the culture medium was removed and the thyrocytes washed twice with 1 ml EBS before fresh DMEM was added (containing no CPSR-5). Selenium, as sodium selenite, was added at concentrations ranging from 10 to 1000 nM to wells in triplicate, in the presence or absence of TSH (1 U/L). After a further 24 h, the medium was removed, the thyrocytes washed again and fresh medium added containing the same concentrations of TSH and selenium as in the initial incubation. The thyrocytes were then cultured for a further 4 days before being washed twice with EBS and removed from the plate as described in Section 4.01c. ID-I activity in the thyrocyte sonicates was measured as previously described in Section 2.06. The protein content of each sonicates sample was also determined and used to correct the ID-I activity for protein (Section 2.07).

b) Effect of different cell culture protocols on selenium status

To investigate whether or not the adopted cell culture protocol could significantly alter selenium status, human thyrocytes were prepared as described in Section 2.03 and grown in 75cm^2 flasks at a density of 10 million cells per flask in 20 ml of growth medium (DMEM/10% CPSR-5). After 24 h the medium was removed from the flasks, and the thyrocytes washed twice with 20 ml of EBS. To half of the flasks, 20 ml of fresh growth media with TSH (DMEM/10% CPSR-5/1 U/L TSH) was added, and the thyrocytes cultured for a further 5 days. The remaining thyrocytes were cultured in 20 ml of serum free growth medium with TSH (DMEM/1 U/L TSH) for 24 h prior to an extra wash protocol consisting of two washes (20 ml per wash). Subsequently, 20 ml of fresh DMEM/1 U/L TSH was added and the thyrocytes cultured for a further 4 days (N.B. total time in culture was equal to those thyrocytes grown with 10% CPSR-5 present). At the end of the incubation period the thyrocytes were harvested as previously described (Section 4.01e) and the cell pellets frozen at -70°C. The GPx and selenium levels were kindly measured by Dr. J.R. Arthur and F. Nicol of the Rowett Research Institute by methods previously described (Beckett *et al,* 1990; Arthur, 1988).

c) Effect of gold thioglucose on T_3 and T_4 production by human and sheep thyrocytes in primary culture

Human and sheep thyrocytes were prepared and grown as described in Section 2.03. After 24 h the medium was removed from the thyrocytes in monolayer culture and the cells washed twice with 1 ml of EBS before 1 ml of fresh growth medium (DMEM/10% CPSR-5) containing KI (10 μM) and TSH (1 U/L) was added. To wells in triplicate, gold thioglucose was added to give a concentration of range from 10^{-9}M to 10^{-4}M. After 5 days incubation the concentration of T_3 and T_4 secreted into the culture medium was determined as described in Section 2.04c.

d) Effect of gold thioglucose on ID-I activity in human thyrocytes

Human thyrocytes were prepared as described in Section 2.03. After 24 h, the culture medium was removed and the thyrocytes washed twice with 1 ml of EBS before fresh growth medium (DMEM/10% CPSR-5) containing TSH (1 U/L) was added. To wells in triplicate gold thioglucose was added over a concentration range of 10^{-9} to 10^{-3} molar. The thyrocytes were then cultured for a further 5 days before being washed three times with EBS and removed from the plate as described in Section 4.01c. ID-I activity in the thyrocyte sonicates was measured as previously described (Section 2.06). The protein content of each well was also measured and used to correct the ID-I activity for protein (Section 2.07).

e) Labelling of selenoproteins in human thyrocytes using [^{75}Se]-selenite

Human thyrocytes were prepared as described in Section 2.03, plated out in four 75cm^2 flasks at a density of 10 million cells per flask in 20 ml of growth medium (DMEM/10% CPSR-5) and subsequently incubated at 37°C with a 5% CO_2 atmosphere. After 24 h, the medium was removed and the cells washed twice with 20 ml of EBS before 0.74 MBq of [^{75}Se]-selenite (specific activity :- 74 MBq/mg) was added to each flask in 20 ml of fresh growth medium. To half of the cells, PMA and A23187 were added, both at a concentration of 1 μM in the presence or absence of TSH (1 U/L). The remaining cells were grown in the absence of PMA/A23187 and in the presence or absence of 1 U/L TSH.

After a further 5 days the growth medium was removed, the thyrocytes washed twice with EBS and then removed from the flasks as described in Section 4.01e. The resulting pellets were then sonicated in 1 ml EBS prior to determination of the protein concentration. Samples were then diluted to a protein concentration of 1 g/L using labelling buffer before being heat treated (90°C, 10 min) with "boiling mix" at a ratio of 2:1 (sample : boiling mix). The samples were loaded (20μg/track) and run on an SDS/PAGE

gel as described in Section 2.09. The resulting gel was dried and autoradiographed as described in Sections 2.09 and 2.10.

f) Investigation of selenium trapping by human thyrocytes

Human thyrocytes were prepared as described in Section 2.03 and maintained in suspension culture at a cell density of $1x10^6$ thyrocytes/ml of growth medium (DMEM/10% CPSR-5) in 50 ml pots. TSH at 1 U/L was added to half the thyrocytes, and all the thyrocytes were pre-incubated for 16 h. Four pots were set up; two were incubated at 37°C for the duration of the experiment, and the other two at 4°C for 18 h (N.B. switched to 4°C 2 h prior to the start of the experiment), with a further 9 h incubation at 37°C. Hence 4 groups were created; a) TSH -, 4°C; b) TSH -, 37°C; c) TSH +, 4°C and d) TSH +, 37°C (+/- TSH denotes presence/absence of 1 U/L TSH). After the pre-incubation, the experiment was started by the addition of [^{75}Se]-selenite (specific activity :- 74 MBq/mg Se) to each pot to give a final concentration of tracer equal to 74 kBq per ml of growth medium. After thorough mixing, 400μl samples in triplicate were removed from each pot into Eppendorf tubes at specified times (0 - 27 h) after the addition of the ^{75}Se-selenite, and centrifuged at 3500 g for 5 minutes to pellet the thyrocytes. Subsequently, the pellets were washed 3 times with 1 ml of isotonic saline (0.9%, w/v), before being counted for the radioactivity resulting from gamma decay of ^{75}Se.

5.02 : Results

a) Effect of selenium on ID-I activity in human thyrocytes

A large degree of variability was observed in the level of ID-I activity in the thyrocytes when exposed to TSH (1 U/L) and the various concentrations of selenium, when comparing thyrocytes from different preparations (figure 5a - 5e). Addition of TSH to the thyrocytes in the absence of selenium resulted in a significant increase in some

experiments (figure 5a - 5c), while in others a small, but not significant increase occurred (figure 5d & 5e). Addition of a range of selenium concentrations (0 - 1000 nM) to the thyrocytes in the absence of TSH failed to cause any significant change in the level of ID-I activity in all 5 experiments (figure 5a - 5e). In contrast, ID-I activity increased in the presence of TSH, as the selenium concentration rose to an optimum concentration of 500 nM in 3 of the experiments (figure 5a - 5c); the other 2 experiments exhibiting a similar pattern though the changes were not significant (figures 5d & 5e). A higher concentration of selenium (1000 nM) resulted in a significant decrease in activity when compared to the optimum concentration of 500 nM, though the level of ID-I activity still remained above the level observed without added selenium, but in the presence of TSH. Overall similar patterns of change were observed in normal (figure 5a), multinodular (figure 5b) and Graves' (figure 5c) derived thyrocytes.

b) Effect of different cell culture protocols on selenium status

The thyrocytes grown in the absence of CPSR-5 (i.e serum free), had a tendency to exhibited lower levels of GPx and selenium than those grown in the presence of CPSR-5 (table 5a), though the differences were not significant (i.e. $p > 0.05$ using an unpaired t-test and a Welch test, respectively).

c) Effect of gold thioglucose on T_3 and T_4 production by human and sheep thyrocytes in primary culture

Gold thioglucose failed to cause any significant change in the amount of T_3 produced by the human thyrocytes in primary culture (figure 5f). In contrast to this, the amount of T_4 produced was slightly decreased by the addition of gold thioglucose, with very small amounts of both thyroid hormones produced (figure 5f). The production of both T_3 and T_4 by sheep thyrocytes was much higher and increased significantly with the addition of

gold thioglucose at concentrations above 10^{-7}M for T_3 and 10^{-5} for T_4 (figure 5g). The maximum observed increases in both thyroid hormones occurred at 10^{-4}M, and consisted of a 65% rise, though even higher increases in T_3 and T_4 concentration might occur at gold thioglucose concentrations in excess of 10^{-4}M.

d) Effect of gold thioglucose on ID-I activity in human thyrocytes

Gold thioglucose at a concentrations at and below 10^{-7}M caused no significant change in the ID-I activity measured in the human thyrocyte sonicates (figure 5h). In contrast, the addition of gold thioglucose at concentrations in excess of 10^{-6} showed significant inhibition of ID-I activity. Maximum inhibition was observed at 10^{-3}M and consisted of a 7-fold decrease in the measured level of ID-I activity when compared to the control (no gold thioglucose).

e) Labelling of selenoproteins in human thyrocytes using [^{75}Se]-selenite

Approximately 17 major bands were labelled with ^{75}Se when the human thyrocytes were grown in the presence of [^{75}Se]-selenite (figure 5i). Eight bands appeared to be labelled with equal intensity in all of the samples, regardless of the additions made. These proteins had approximate molecular weights of 15.0, 18.0, 20.0, 21.5, 22.5, 25.0, 26.5 and 56.0 kDa. In addition to these, the labelling of other proteins varied according to the additions made. TSH had no noticeable effect on the expression of most of the proteins, the exception being ID-I (M.W $\approx$ 28.1). The expression of ID-I increased with TSH, but was decreased significantly in the presence of PMA and A23187. The expression of 3 other bands also decreased on addition of PMA and A23187 (M.Ws $\approx$ 24.0, 66.2 & 72.5 kDa). PMA and A23187 did however induce the expression of other proteins with approximate molecular weights of 31.7, 51.5, 58.5, 63.0 and 97.4 kDa. The 58.5 kDa protein was also present in thyrocytes grown without PMA and A23187, regardless of the

presence or absence of TSH. The intensity of this band however, was much higher when PMA and A23187 were present.

f) Investigation of selenium trapping by human thyrocytes

A significant amount of [^{75}Se]-selenite was trapped by thyrocytes after 27 h of incubation, regardless of the manipulations made (figure 5j). TSH had no significant effect on the rate of ^{75}Se trapping or total amount of ^{75}Se trapped by the human thyrocytes after 27h. There was a clear, significant difference in the rate of ^{75}Se trapping between the groups incubated at 37°C or 4°C. Those thyrocytes incubated at 37°C trapped approximately 60,000 cpm of ^{75}Se, and reached a plateau after 12 h. In contrast, those maintained at 4°C trapped only 8,000 cpm in the same period of time, but on further incubation at 37°C they increased the rate of trapping such that both groups had trapped the same amount of ^{75}Se (≈ 60,000 cpm) after 27 h (figure 5j).

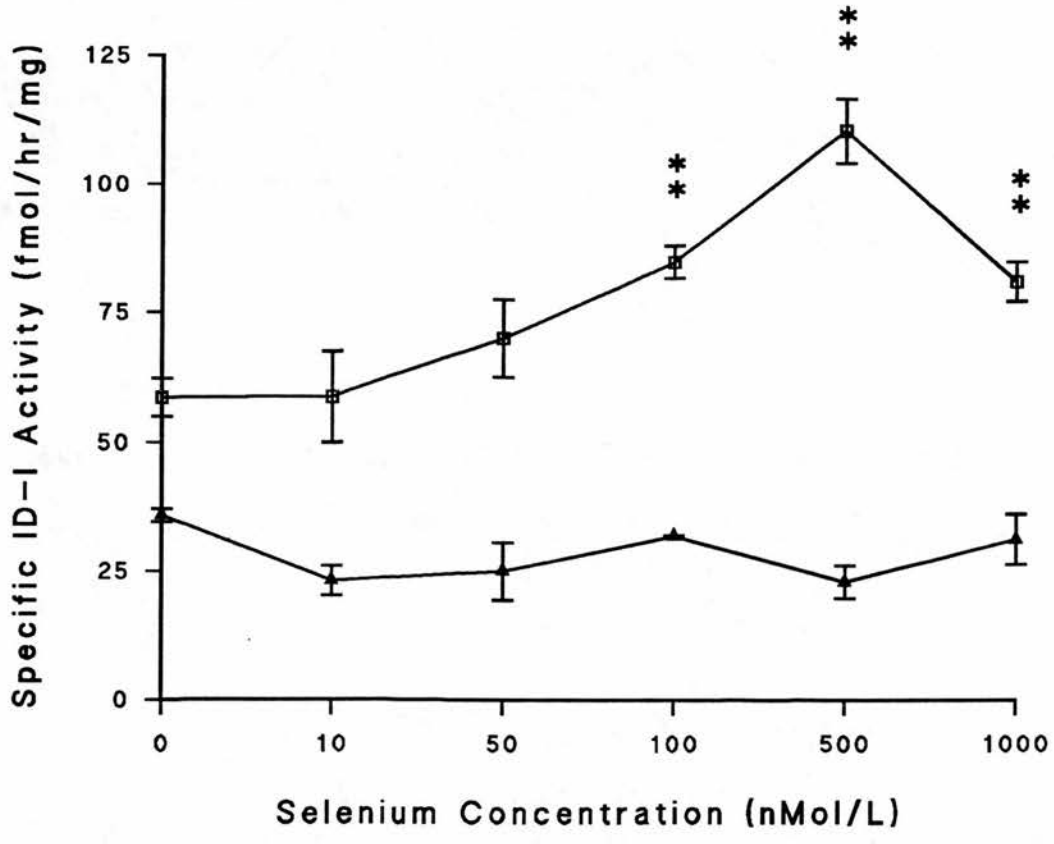


Figure 5a : Effect on ID-I activity of adding selenium to the medium of human thyrocytes isolated from normal tissue, grown in the presence (□) or absence (Δ) of TSH (1 U/L) for 5 days. Values shown are the mean ± SEM of wells in triplicate. TSH caused a significant increase in ID-I activity ($p < 0.05$), with a further significant rise at selenium concentrations of 100 nM or greater. Selenium at a concentration of 1000 nM caused a significant decrease in ID-I activity compared to 500 nM Se ($p < 0.01$).

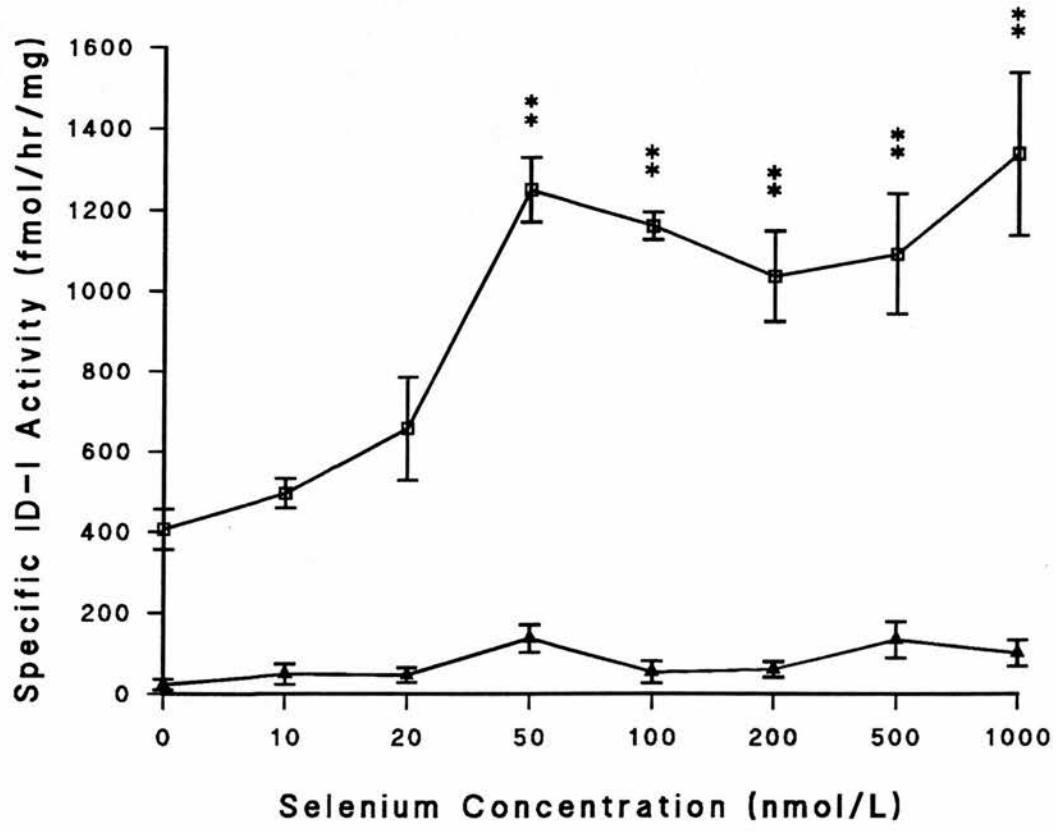


Figure 5b : Effect on ID-I activity of adding selenium to the medium of human thyrocytes isolated from multinodular hyperthyroid tissue, grown in the presence (□) or absence (Δ) of TSH (1 U/L) for 5 days. Values shown are the mean ± SEM of wells in triplicate, with a significant increase observed in the presence of TSH at selenium concentrations of 50 nM or greater. TSH also caused a significant increase in ID-I activity in the absence of selenium ($p < 0.001$).

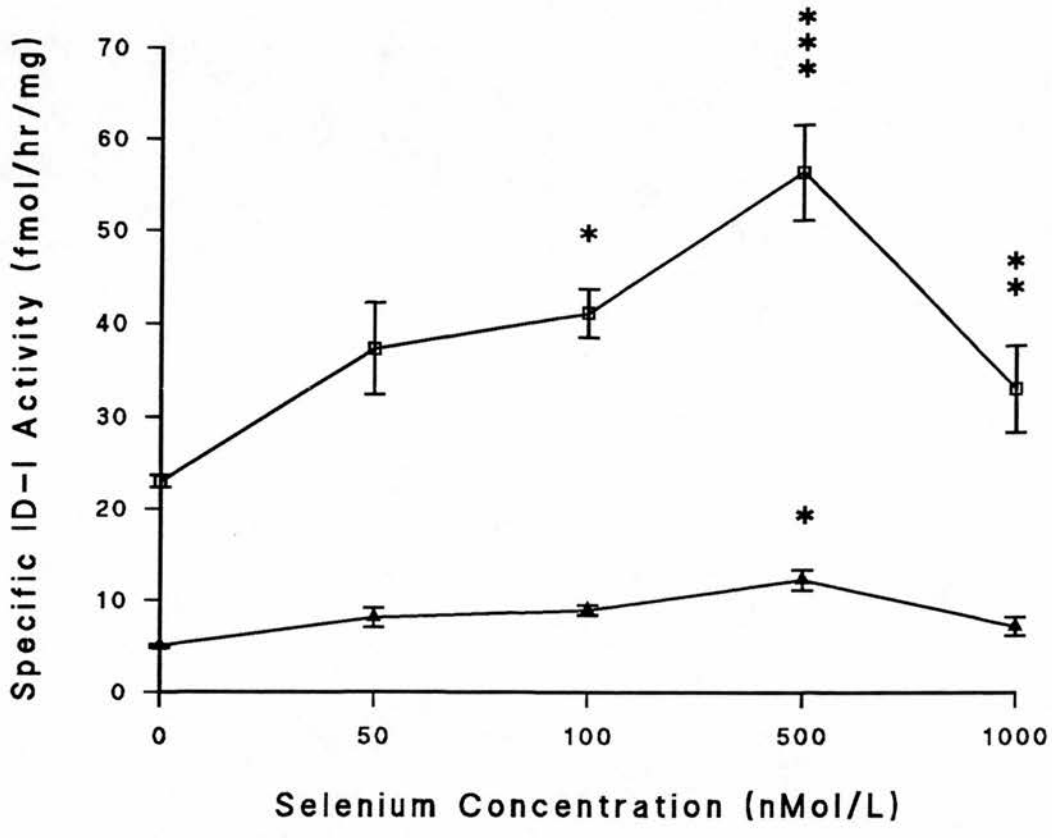


Figure 5c : Effect on ID-I activity of adding selenium to the medium of human thyrocytes isolated from Graves' hyperthyroid tissue, grown in the presence (□) or absence (Δ) of TSH (1 U/L) for 5 days. Values shown are the mean ± SEM of wells in triplicate. Significant increases in ID-I activity occurred at 100 and 500 nM selenium, in the presence of TSH. A significant decrease in ID-I occurred between 500 and 1000 nM selenium ($p < 0.01$). TSH caused a significant increase in ID-I activity in the absence of selenium ($p < 0.01$).

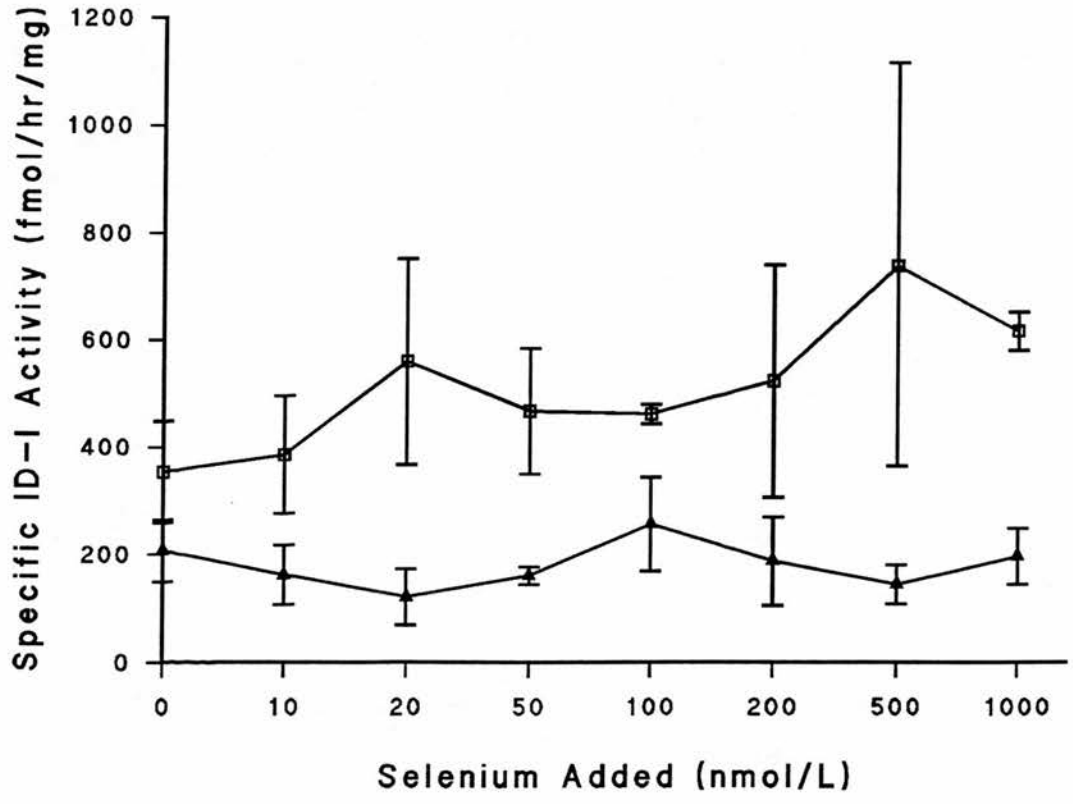


Figure 5d : Effect on ID-I activity of adding selenium to the medium of human thyrocytes isolated from Graves' hyperthyroid tissue, grown in the presence (□) or absence (Δ) of TSH (1 U/L) for 5 days. Values shown are the mean ± SEM of wells in triplicate. No significant changes were observed.

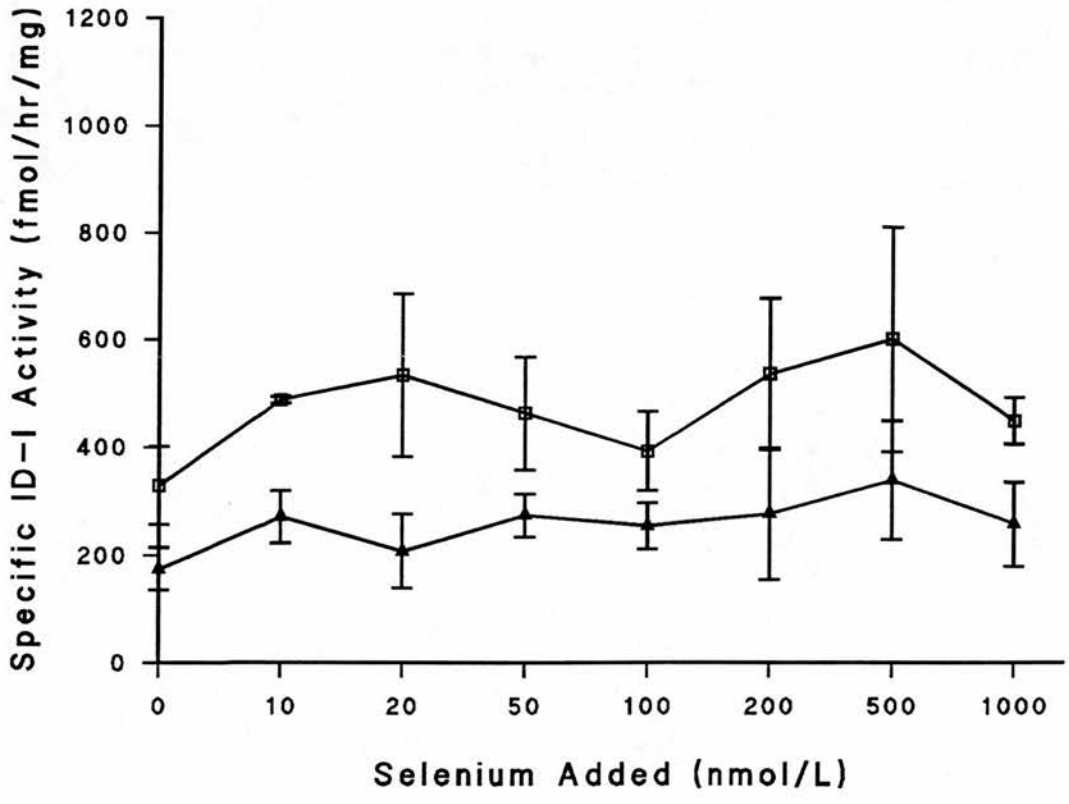
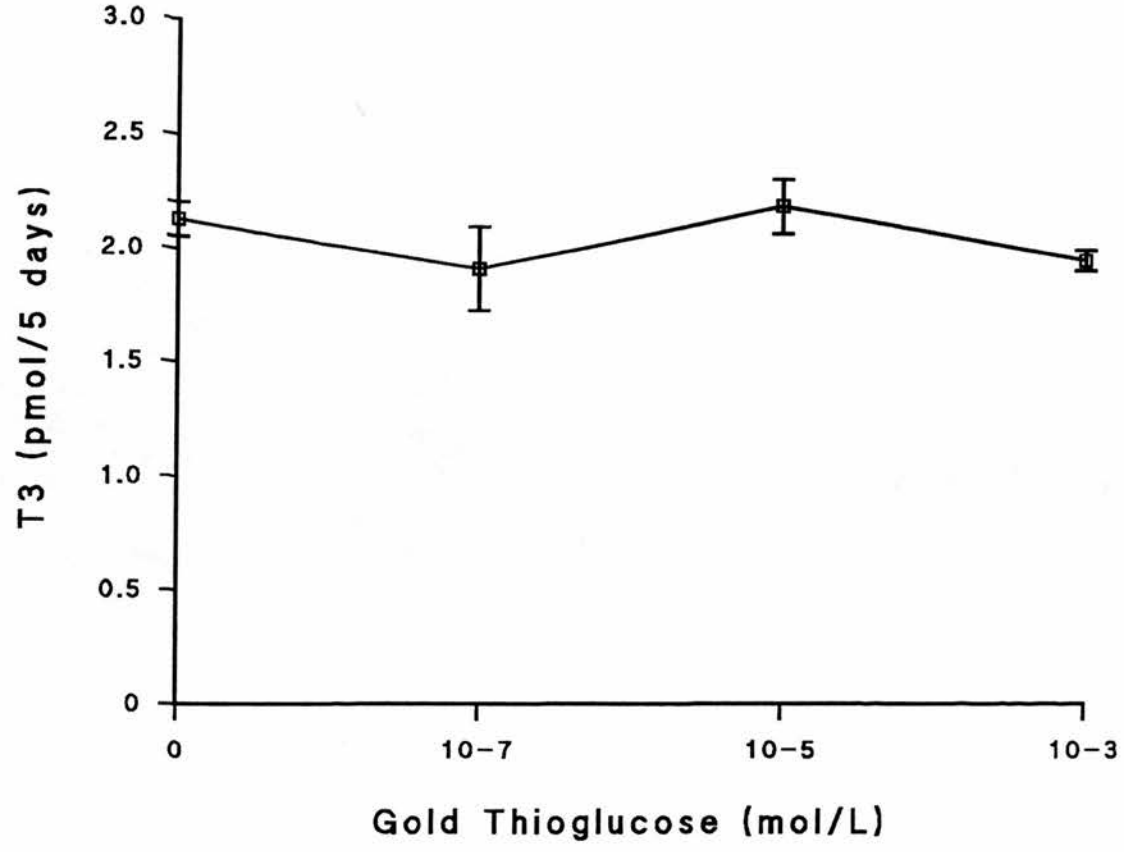


Figure 5e : Effect on ID-I activity of adding selenium to the medium of human thyrocytes isolated from Graves' hyperthyroid tissue, grown in the presence (□) or absence (Δ) of TSH (1 U/L) for 5 days. Values shown are the mean ± SEM of wells in triplicate. No significant changes were observed.

Table 5a : Level of selenium and GPx activity in human thyrocytes grown in the presence or absence of 10% CPSR-5 (fetal calf serum). Thyrocytes grown in the absence of CPSR-5 were also subjected to an extra washed in an attempt to deplete the thyrocytes of selenium. No significant changes occurred. These measurements were kindly performed by Dr. J.R. Arthur and F. Nicol of the Rowett Research Institute, Aberdeen, U.K.

Culture Protocol	**GPx Activity** (U/mg, n = 6)	**Selenium Level** (ng/mg, n = 3)
DMEM only	0.035 ± 0.005	1.55 ± 0.52
DMEM/10% CPSR-5	0.045 ± 0.002	1.83 ± 0.11

Figure 5f : Effect of 5 days exposure to gold thioglucose on thyroid hormone production from human thyrocytes grown in primary culture in the presence of TSH (1 U/L). The accumulated medium content of T_3 (□) and T_4 (Δ) was measured and the data expressed as mean ± SEM of triplicate wells in a representative experiment performed on at least three occasions. Significant decreases in T_4 accumulation occurred with gold thioglucose at 10^{-7}M and 10^{-5} M when compared to the control (i.e. - gold thioglucose).


3.0
2.5
2.0
1.5
1.0
0.5
0
T3 (pmol/5 days)
0
10-7
10-5
10-3
Gold Thioglucose (mol/L)

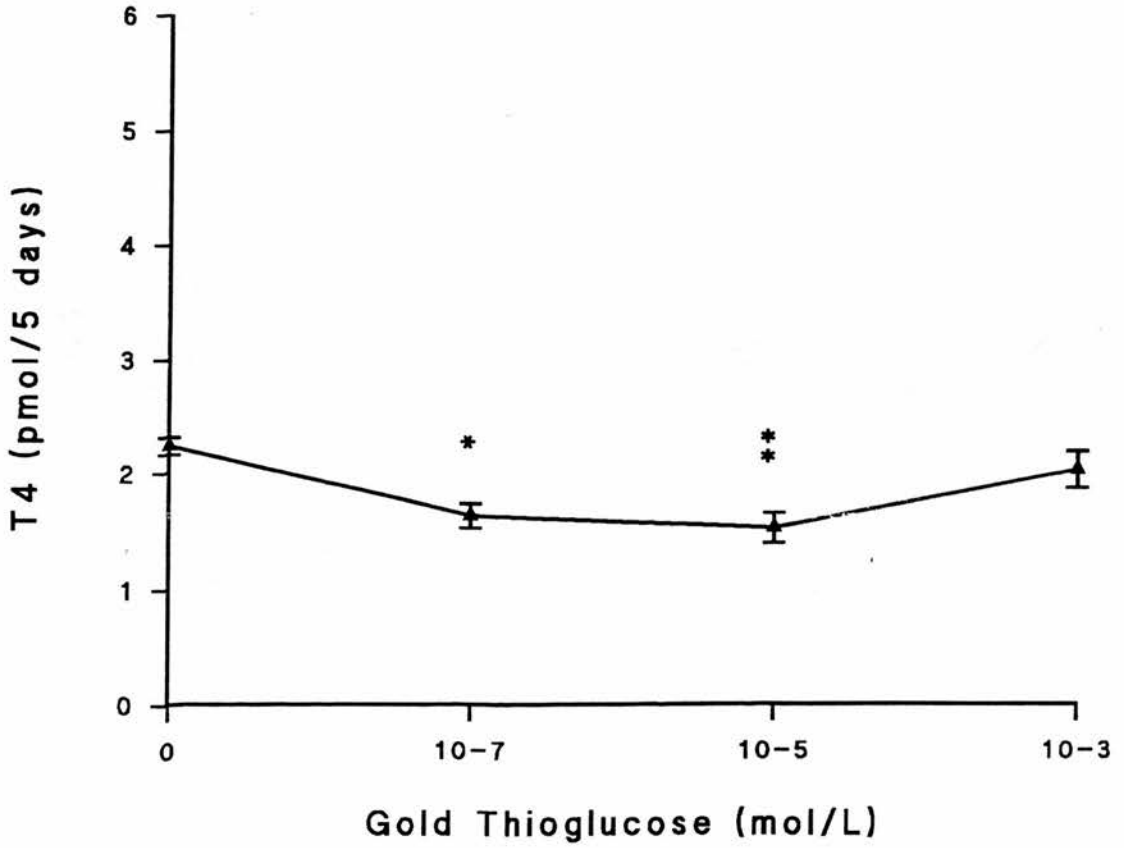
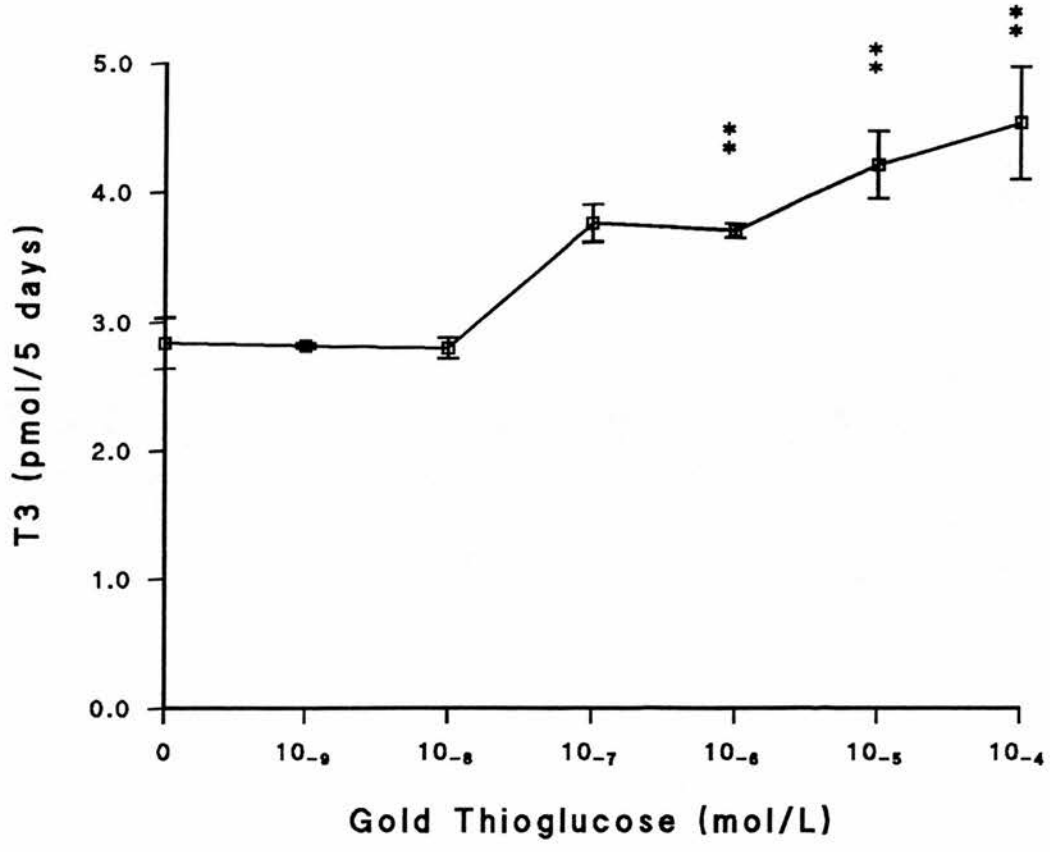
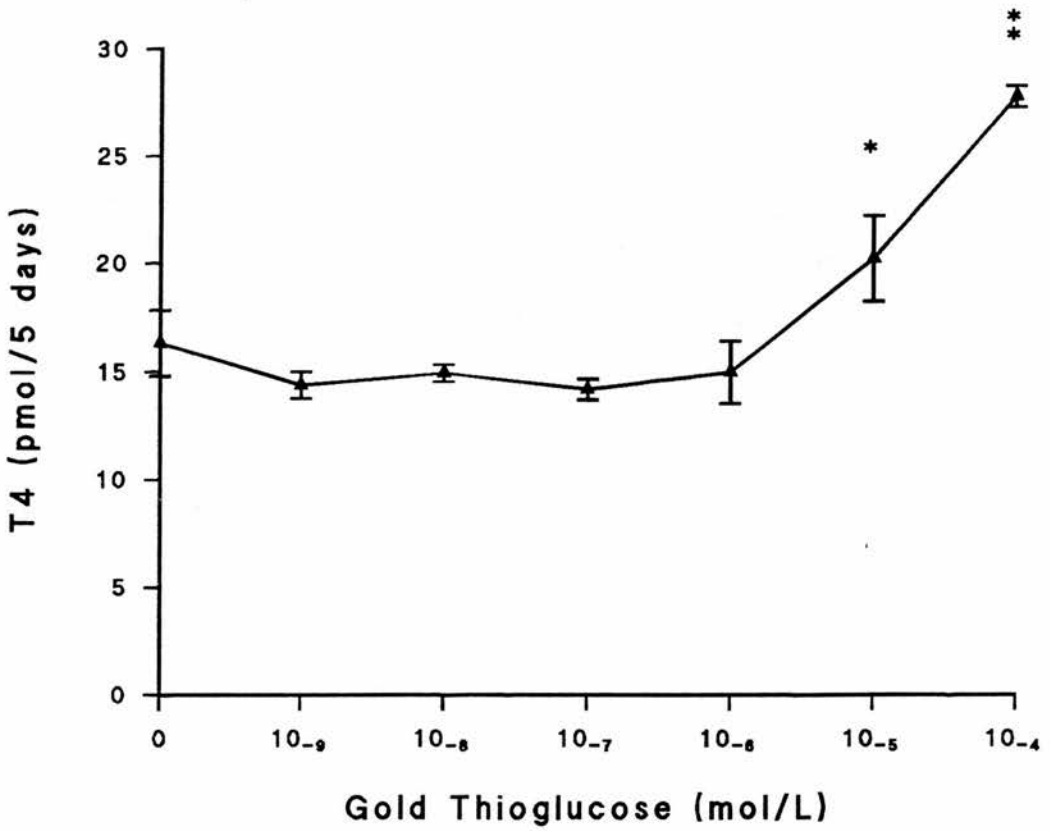

6
5
4
3
2
1
0
T4 (pmol/5 days)
*
**
0
10-7
10-5
10-3
Gold Thioglucose (mol/L)

Figure 5g : Effect of 5 days exposure to gold thioglucose on thyroid hormone production from sheep thyrocytes grown in primary culture in the presence of TSH (1 U/L). The accumulated medium content of T_3 (□) and T_4 (Δ) was measured and the data expressed as mean ± SEM of triplicate wells. Significant increases in T_3 and T_4 accumulation occurred at gold thioglucose concentrations of 10^{-7} or greater when compared to the control (i.e. - gold thioglucose).


T3 (pmol/5 days)
5.0
4.0
3.0
2.0
1.0
0.0
0
10-9
10-8
10-7
10-6
10-5
10-4
Gold Thioglucose (mol/L)


T4 (pmol/5 days)
30
25
20
15
10
5
0
0
10-9
10-8
10-7
10-6
10-5
10-4
Gold Thioglucose (mol/L)

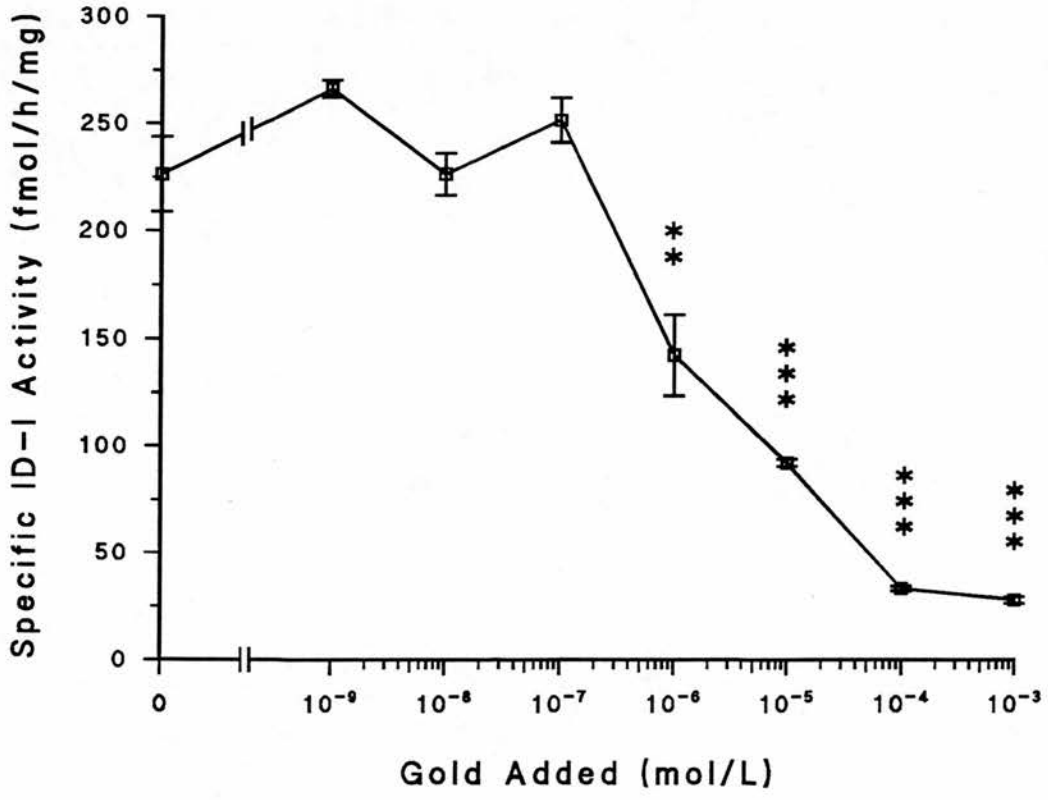


Figure 5h : Effect on ID-I activity of adding gold thioglucose to human thyrocytes grown in primary culture in the presence of TSH (1 U/L) for 5 days. Values shown are the mean ± SEM of triplicate wells in a representative experiment carried out on three occasions. Concentrations of gold thioglucose in excess of 10^{-6}M caused significant decreases in ID-I activity.

Figure 5i : Autoradiography of an SDS-PAGE gel of thyrocyte sonicates labelled with ^{75}Se during growth in the presence or absence of various compounds. Thyrocytes were grown in the presence (tracks 3 & 4) and absence (track 1 & 2) of TSH (1 U/L), and also in the presence (tracks 2 & 4) and absence (tracks 1 & 3) of PMA/A23187 (10^{-6}M). Track 5 shows affinity labelled human liver microsomes used as an aid in determining the identity of the ID-I band. The mobilities of the molecular mass markers and ID-I are also indicated.

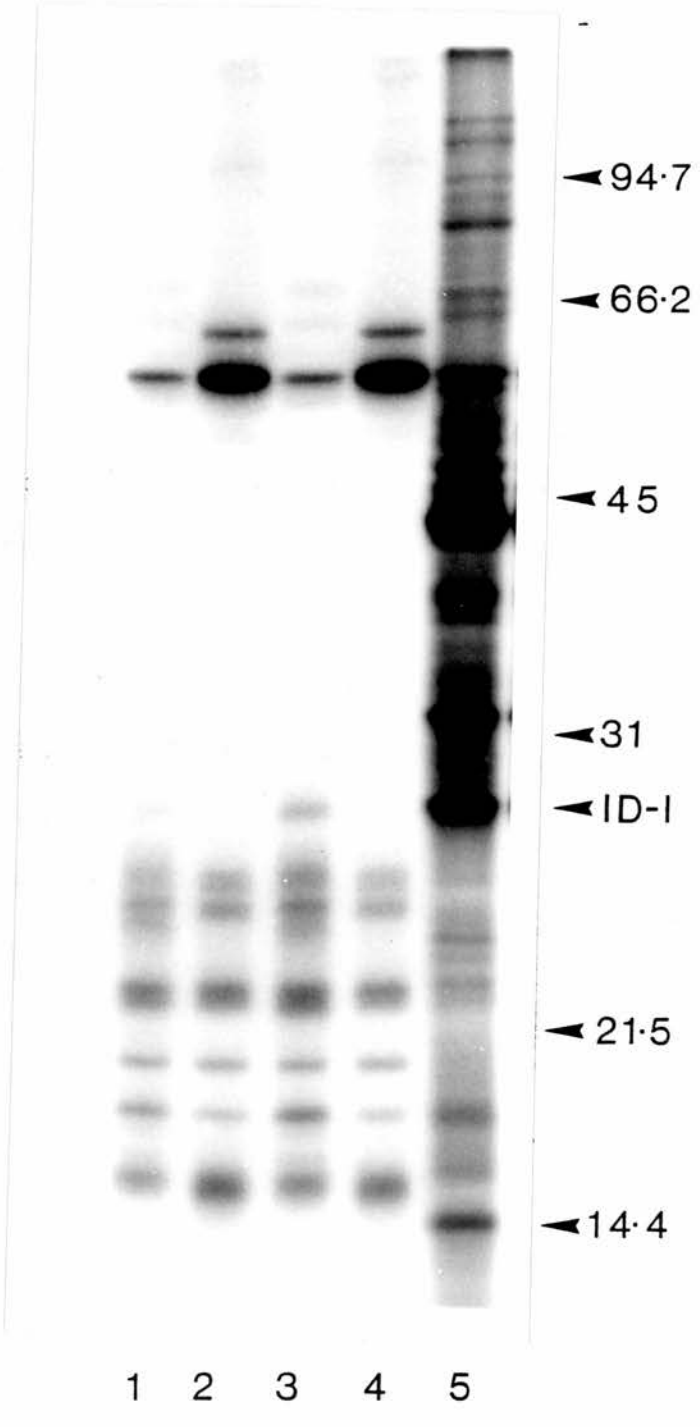
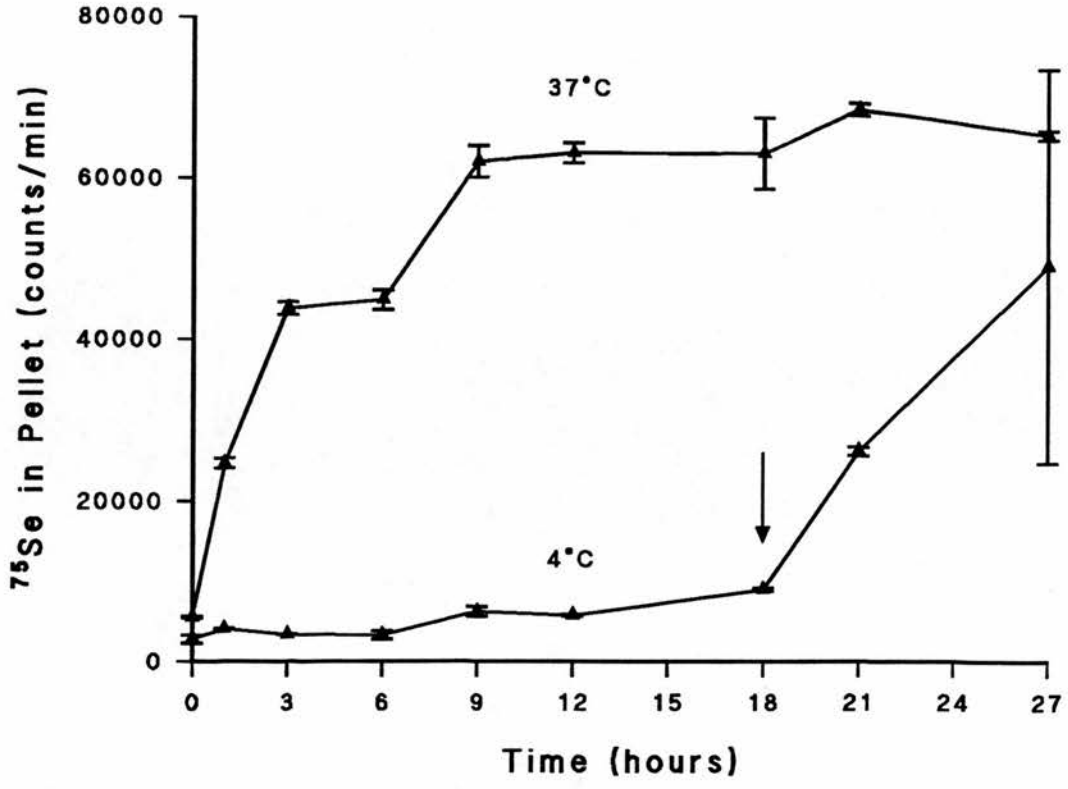
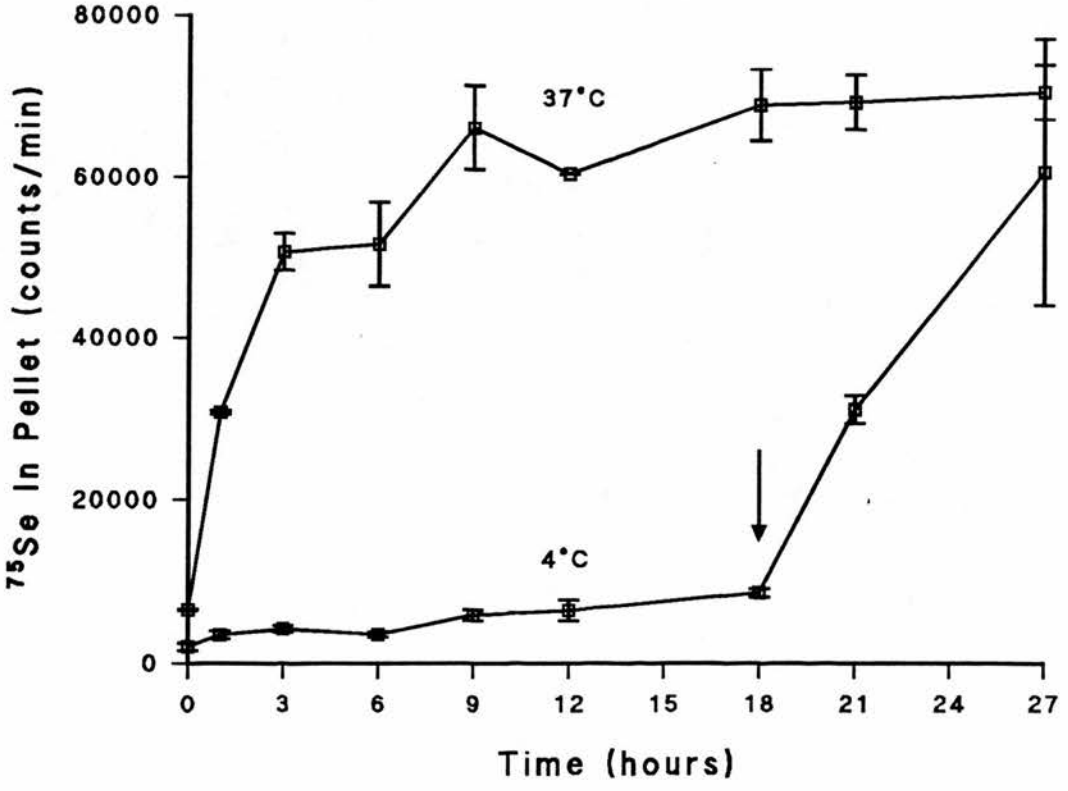

94·7
66·2
45
31
ID-I
21·5
14·4
1
2
3
4
5

Figure 5j : Effect of temperature and TSH on ^{75}Se trapping in human thyrocytes pelleted by centrifugation after incubated in suspension culture in the presence of 74kBq/ml of ^{75}Se. Thyrocytes incubated at 4°C were switched to 37°C after 18 h (denote by ↓). Thyrocytes were grown either in the presence (□) or absence (Δ) of TSH (1 U/L). Values shown are mean ± SEM for samples taken in triplicate for a representative experiment carried out on three occasions. At 37°C, time points above 1 hr showed significant trapping of ^{75}Se in the presence and absence of TSH. Until the thyrocytes were switched to 37°C, the thyrocytes incubated at 4°C showed no significant trapping of ^{75}Se, but had statistically trapped the same amount of ^{75}Se at 27h.


80000
60000
40000
20000
0
^{75}Se in Pellet (counts/min)
37°C
4°C
0
3
6
9
12
15
18
21
24
27
Time (hours)


80000
60000
40000
20000
0
^{75}Se In Pellet (counts/min)
37°C
4°C
0
3
6
9
12
15
18
21
24
27
Time (hours)

5.03 : Discussion

TSH added at a concentration of 1 U/L induced an increase in ID-I activity, even in the absence of added selenium (figures 5a - 5e). In the absence of TSH, the addition of increasing amounts of selenium had no significant effect on ID-I activity (figures 5a - 5e). In contrast, in most thyrocyte preparations a small, dose-dependent increase (2 or 3-fold) in ID-I activity could be seen as selenium was added to the thyrocytes grown in the presence of TSH. These data suggest that thyrocytes retained sufficient selenium to allow a limited increase in ID-I expression. Indeed, the data produced subsequently (table 5a) demonstrates that the thyrocytes retained significant levels of selenium and GPx activity under the culture conditions used, though the levels of both selenium and GPx had a tendency to be slightly lower in the absence of CPSR-5, than in the presence of 10% CPSR-5. This data, in conjunction with the observed increases in ID-I activity implies that selenium supply may be slightly inadequate under these conditions for normal levels of selenoprotein expression.

Other workers have also shown a dependence of ID-I on selenium concentration using cells in culture (Oertel *et al,* 1993). Using the porcine-kidney cell line (LLC-PK1) these workers demonstrated a clear dependence of ID-I activity on selenium, with a plateau observed at 10 nmol selenite/L. Clearly there is a significant difference between the LLC-PK1 and primary cultured human thyrocyte systems with respect to the concentration of selenite required for maximum ID-I activity (10 nM and 500 nM respectively). The cause of this difference is unclear, however this difference may be an artefact of using cell lines. Cell lines often behave differently than primary cultured cells; for example the human thyroid cell lines HTori-3 and FTC-133 lack ID-I expression, while primary cultures of multinodular, normal and Graves' human thyroid tissues all express significant levels of this enzyme (Beech, 1992; Ishii *et al,* 1981; Oertel *et al,* 1991). I also observed with the human thyrocytes that selenite at a concentration of 10 μM or greater

was cytotoxic to human thyrocytes, while Oertel and coworkers show similar, cytotoxic effect of selenite at the slightly lower concentration of 5 μM. Oertel *et al*, also found that the full expression of glutathione peroxidase (GPx) required 10 - 20-fold higher concentrations of selenite than for the full expression of ID-I. My data and that of Oertel *et al* clearly demonstrates that ID-I levels are maintained or can even be increased in selenium-deficient conditions, while the level of GPx expression decreases. These findings support the hypothesis of Behne *et al*, who proposed the existence of a molecular hierarchy for selenium supply, with selenoproteins other than glutathione peroxidase receiving a preferential supply of selenium (Behne *et al,* 1988).

Addition of gold thioglucose to human thyrocytes grown in the presence of TSH and KI caused no significant change in the level of T_3 produced by the thyrocytes, but a small, significant decrease in T_4 production was noted (figure 5f). Since gold thioglucose has been shown to inhibit selenoenzymes by binding to the selenocysteine residue found at the active site (Chaudiere and Tappel, 1984), it might be expected that the addition of gold thioglucose would cause an increase in T_4 production and a decrease in T_3 production by inhibiting ID-I activity. Indeed, gold thioglucose does inhibit ID-I as described below, and hence the reason for the lack of a gold effect on thyroid hormone production is unclear at present. In contrast to the human thyrocytes, addition of gold thioglucose to sheep thyrocytes produced large changes in the amount of thyroid hormones found in the culture medium (figure 5g). Production of T_3 and T_4 were both significantly increased when gold thioglucose was added at concentrations above 10^{-6}M. This increase in thyroid hormone production may be mediated by an inhibition of glutathione peroxidase, the selenoenzyme responsible for protecting the thyrocytes against peroxide damage by hydrogen peroxide. Inhibition of GPx could result in a significant increase in the intracellular concentration of hydrogen peroxide. The availability of hydrogen peroxide has been shown to be the rate limiting step in the organification of

iodine by thyroperoxidase (TPO) and hence thyroid hormone synthesis (Ahn and Rosenberg, 1970; Bjorkman and Ekholm, 1988). Thus elevated levels of intracellular hydrogen peroxide could increase organification of iodine and hence increase synthesis of both thyroid hormones.

The addition of gold thioglucose to human thyrocytes grown in the presence of TSH (1 U/L) resulted in a significant decrease in the level of ID-I activity measured in the thyrocyte sonicates (figure 5h). At concentrations below 10^{-7}M, gold thioglucose caused no significant change in ID-I activity, while concentrations in excess of 10^{-6}M resulted in large decreases in ID-I activity. Maximum inhibition of ID-I occurred at 10^{-3}M and consisted of a 88% decrease. Berry *et al* have also shown inhibition of ID-I by gold thioglucose, with a 40% inhibition observed at 10 nM *in vitro* (Berry *et al,* 1991b). Furthermore, these workers have shown that gold thioglucose at 100-fold higher concentrations can inhibit a mutant form of ID-I, consisting of cysteine substituted for selenocysteine, and thereby confirm that gold thioglucose acts by binding to the reactive selenium of the selenocysteine found at the active site.

When human thyrocytes were grown in the presence of [^{75}Se]-selenite, approximately 17 major [^{75}Se]-labelled bands were visible on an autoradiograph (figure 5i). These proteins had approximate molecular weights of 15.0, 18.0, 20.0, 21.5, 22.5, 24.0, 25.0, 26.5, 28.3, 31.7, 51.5, 56.0, 58.5, 63.0, 66.2, 72.5 and 97.4 kDa. Eight proteins were equally expressed by all of the thyrocytes, regardless of the various additions made to the growth medium (M.Ws ≈ 15.0, 18.0, 20.0, 21.5, 22.5, 25.0, 26.5 and 56.0 kDa). The identity of most of these proteins is unknown, however some of these proteins have been previously shown to be expressed in various rat tissues (Behne *et al,* 1988) and hence they are not specific to the human thyroid. The expression of the remaining 9 proteins was modified by the addition of PMA/A23187 (both at 1 μM) and/or TSH (1 U/L) to the culture medium. TSH had no noticeable effect on the expression of most of the labelled

proteins with the exception of ID-I (M.W. ≈ 28.1). In the presence of PMA and A23187 the ID-I band disappeared, as did 3 other bands with approximate molecular weights of 24.0, 66.2 & 72.5 kDa. PMA and A23187 also induced the expression of several other proteins with molecular weights of 31.7, 51.5, 58.5, 63.0 and 97.4 kDa. Induction of the 58.5 kDa protein band was marked, however unlike the other proteins induced by PMA/A23187 this 58.5 kDa protein was also present in thyrocytes grown in the absence of PMA/A23187.

The existence of selenoproteins with molecular weights of 58, 56 and 14 kDa has been reported by various members of Medina's group (Morrison *et al,* 1988b; Morrison *et al,* 1988a; Bansal *et al,* 1989b; Bansal *et al,* 1990b; Bansal *et al,* 1990a). These workers showed that the 58 kDa selenoprotein could mediate the growth inhibitory effects of high selenium in murine mammary epithelial cells, or at least that its expression correlated well with the selenium sensitive mechanisms which could control growth. In addition these workers have showed that higher levels of this selenoprotein were present in cells which were not dividing, when compared to cells undergoing rapid cell division, with evidence for inhibition of DNA synthesis by this protein (Morrison *et al,* 1988b; Morrison *et al,* 1988a).

The 56 kDa and 14 kDa selenoproteins (now termed SP56 & SP14) were also investigated by Medina's group (Bansal *et al,* 1989b), and are distinct from the 58 kDa protein (Lanfear *et al,* 1993). Expression of the SP56 and SP14 proteins in various rodent tissues was shown by Medina and coworkers to be independent of dietary selenium, with the DNA for the SP56 and SP14 proteins also shown to lack an in-frame TGA codon which could code for a selenocysteine residue (Bansal *et al,* 1990b; Bansal *et al,* 1990a). Hence, these proteins are not like the selenoproteins characterised in recent years (e.g. GPx, selenoprotein P, ID-I etc.) which contain a selenocysteine residue. It has been suggested that selenium mediates it's effects by modifying the function of pre-existing proteins, two of which may be SP56 and SP14 (Bansal *et al,* 1990b). Wether the function

of the 58 kDa selenoprotein is mediated in a similar manner is not known at present. However, the addition of methionine and serine together has been shown to increase the expression of the 58 kDa protein, with this increase postulated to occur in response to enhanced provision of selenocysteine (Morrison *et al,* 1988a). This observation implies that the 58 kDa protein contains a selenocysteine specifically incorporated in response to a UGA codon. The way in which selenium is associated with the SP56 and SP14 selenoproteins is unclear at present, however several proteins retained ^{75}Se during and after electrophoresis on an SDS-PAGE gel and hence the selenium must be tightly bound to these proteins.

The selenoprotein, SP14 is now thought to be the fatty acid binding protein (FABP), found in liver (Bansal *et al,* 1989a). Furthermore, due to the homology between FABP and two growth inhibition proteins found in bovine mammary glands and in fibroblasts, FABP has now be postulated to be involved in the control of cell growth. The role of SP56 is still not fully elucidated. Initially, SP56 was thought to have a role as a selenium transport protein or as growth regulation protein (Bansal *et al,* 1989b). However, it has been demonstrated recently that the SP56 protein has a high degree of sequence homology with the 56 kDa, acetaminophen-binding protein (AP56) (Lanfear *et al,* 1993). The AP56 protein has been shown to protect against acetaminophen-induced hepatoxicity, possibly by scavenging toxic electrophiles or acetaminophen metabolites. Due to structural similarities, SP56 and AP56 are believed to have the same role. Indeed, there is evidence to suggest that the detoxification of acetaminophen is dependent on selenium status, though there is no direct evidence as yet for selenium-dependent acetaminophen-binding to SP56 or AP56 (Lanfear *et al,* 1993).

The regulation of thyrocyte growth is believed to be under the control of all 3 main second messenger cascades (Raspe and Dumont, 1992). As a generalisation, in the thyrocyte the cAMP cascade is believed to cause proliferation and differentiation. The

tyrosine kinases which are usually associated with receptors also bring about thyrocyte growth, but cause dedifferentiation. Finally, the Ca^{2+}-PI cascade appears to have limited effects on growth and differentiation, but also causes an increase in protein synthesis, with a pattern similar to that of tyrosine kinase. Overall, the 3 second messenger cascades are believed to cause changes in the activity and/or expression of various, unknown growth regulatory proteins (Dumont *et al,* 1992). The 58 kDa protein described by Medina's group may well be one of these proteins, with its function dependent on selenium supply. I have shown that PMA and A23187, which simulate the effects of activating the Ca^{2+}-PI cascade caused an increase in the band intensity of a 58.5 kDa selenoprotein in human thyrocytes (figure 5g). This increase may be a result of increased expression, though increased binding of ^{75}Se to the pre-existing protein can not be ruled out unless the mRNA from the protein is isolated and sequenced. Which of these mechanisms is in operation is unclear, though it is intriguing to speculate that the 58.5 kDa protein may be the 58 kDa protein reported by Medina's group to modify cell growth. Changes in this 58.5 kDa protein might also explain the morphological changes observed on addition of PMA and A23187 to human thyrocytes in primary culture. These changes consisted of the disappearance of TSH induced follicles (figure 4f).

I have also demonstrated temperature-dependent accumulation/trapping of [^{75}Se]-selenite in human thyrocytes in primary culture (figures 5j) which was not significantly influenced by the presence of TSH in the growth medium. As the accumulation of selenite was temperature-dependent, the mechanism responsible is most probably energy-dependent. However, the experimental method does not distinguish between an active transport mechanism or trapping/incorporation of selenite in selenoproteins. Other studies offer support for an active transport mechanism, and these including the observation that selenite can been be transported across the brush border membrane of small intestine mucosal cells (Wurmli *et al,* 1989). Wurmli *et al* demonstrated that selenium (as selenite)

can be transported across the brush border membrane as either selenotrisulphides or selenopersulphides by Na^+-dependent amino acid carriers after the reaction of selenite with thiols such as cysteine or glutathione. As there would be an insufficient concentration of thiol compounds within the growth medium, this mechanism is unlikely to be involved in selenite uptake by human thyrocytes. Other workers have shown that erthyrocytes can take up selenium, using selenite in preference to selenate or selenomethionine (Lee *et al,* 1969; Jenkins and Hidiroglou, 1972; Sandholm, 1973a; Sandholm, 1973b). However, this "active" uptake of selenite was found to result from the modification of selenite and subsequent secretion of the product from the erthyrocyte, which thereby maintained a concentration gradient of selenite into the erthyrocyte (Lee *et al,* 1969). Whether selenite can be actively taken up or not, selenite is not normally found to any extent in the body and hence it is not the physiological form of selenium which could possibly be taken up by the thyroid *in vivo*. However, it is of interest that selenium supplementation is usually performed with selenite, and hence selenite trapping may be of importance under these conditions. Most naturally occurring dietary selenium is found as selenomethionine or selenocysteine, with L-selenomethionine shown to be actively transported in the intestine of hamsters by the methionine transport carrier (McConnell and Cho, 1966).

The thyroid gland may also possess a methionine transport system, as TSH has been shown to modify the intrathyroidal cytosolic pools of methionine (Cooper *et al,* 1986). One could speculate that thyroidal uptake of selenomethionine could occur via a methionine transport pathway, with this system stimulated by TSH. The ability of TSH to maintain a supply of selenium to the thyrocyte in selenium deficiency is desirable since TSH would be expected to increase intrafollicular H_2O_2 concentrations. The increase in selenium supply would allow expression of GPx to be maintained and thus protect against peroxidative damage.

A selenomethionine or selenite uptake mechanism could possibly explain the ability of human thyrocytes in primary culture to increase ID-I activity, even in the absence of exogenous selenium by recovering selenium lost to the medium. The presence of a selenium uptake system would also explain the reported ability of the thyroid to retain selenium far longer than liver and kidney (Behne *et al,* 1988).

In summary, the data presented in this Section demonstrates that ID-I is indeed as selenoenzyme, dependent on selenium supply for its expression and activity. However, ID-I is only one of several selenoproteins present in human thyrocytes. One of these selenoproteins with a molecular weight of ≈ 58.5 kDa may have a role in the selenium-mediated regulation of cell growth and division. Finally, thyrocytes could possess an active uptake mechanism for selenium which would allow the thyroid to retain selenium in preference to liver and kidney.

Section 6 : EFFECTS OF SELENIUM AND IODINE DEFICIENCY ON THYROID HORMONE METABOLISM IN RATS

Iodine is an essential trace element for the production of thyroid hormones. Deficiency of this element has been shown to have wide ranging effects on the mental and physical development of the child and fetus, with severe iodine deficiency resulting in cretinism (Hetzel, 1983). Iodine deficiency in adults also has profound effects, but these are restricted to irregularities in the control of metabolism. There are a number of mechanisms which can protect people from the harmful effects of iodine deficiency which include increased iodine trapping by the thyrocytes, and an increase in the proportion of thyroid hormone secreted from the thyroid as triiodothyronine (T_3) (Adams and Larsen, 1973). As a result the plasma T_3 concentration is maintained at the expense of plasma thyroxine (T_4) (Silva, 1985).

Iodine deficiency can also increase the activity of thyroidal ID-I, and hence this enzyme may contribute significantly to the amount of T_3 secreted directly by the thyroid (Green, 1978; Laurberg, 1980; Laurberg, 1984). As ID-I is selenium-dependent, it has been suggested that selenium deficiency can exacerbate the effects of iodine deficiency in rats (Arthur *et al,* 1990c). Dual deficiency of selenium and iodine has also been suggested to be involved in the pathogenesis of endemic cretinism (Goyens *et al,* 1987; Vanderpas *et al,* 1990).

In this section the effects of selenium, iodine and combine selenium/iodine deficiency on ID-I, and plasma thyroid hormones are studied using the rat as a model.

6.01 : Methods

a) Effect of selenium status on hepatic ID-I activity, plasma T_3 and plasma T_4

Male, Hooded Lister, weanling rats of the Rowett Institute strain were divided into six groups and offered diets for 6 weeks, supplemented where appropriate with selenium as follows : a) 0.005, b) 0.015, c) 0.025, d) 0.045, e) 0.1, and f) 0.4 mg selenium per kg of diet mix (as sodium selenite). The rats were given free access to distilled water and food. The food intake was recorded daily, with the body weight of animals recorded on a weekly basis. At the end of the experiment, rats were anaesthetized with diethyl ether, and blood taken into heparinized tubes by cardiac puncture. Livers were perfused with 0.15M KCl at 4°C, removed and immediately frozen in liquid nitrogen.

Plasma T_3 and T_4 concentrations were assayed using the in-house radioimmunoassay as described in Section 2.04c. The hepatic ID-I activities of the various animals were determined in 20% homogenates as described in Section 2.05a.

b) Effect of selenium deficiency on thyroidal ID-I and GPx activities

Male, Hooded Lister, weanling rats of the Rowett Institute strain were divided into two groups, one offered a selenium free diet (Se < 0.005 mg/kg of diet mix) and the other a selenium supplemented diet (0.1 mg Se/kg of diet mix) for 6 weeks (selenium added as sodium selenite). The rats were allowed free access to distilled water and food. At the end of the experiment, the rats were anaesthetized with diethyl ether and the thyroids were removed and immediately frozen in liquid nitrogen.

Glutathione peroxidase activities were measured at the Rowett Research Institute, Aberdeen, by Dr. J.R. Arthur and F. Nicol as previously described (Beckett *et al,* 1990). The thyroidal ID-I activities were assayed using the non-isotopic T_4 method described in Section 2.05b.

c) Effect of selenium, iodine and combined selenium/iodine deficiency on ID-I

Male, Hooded Lister, weanling rats of the Rowett Institute strain were divided into four groups and offered one of the following diets for 6 weeks : i) selenium and iodine supplemented control (Se+ I+), ii) selenium-deficient (Se- I+), iii) iodine-deficient (Se+ I-), and iv) selenium and iodine deficient (Se- I-). The basal diet (Se- I-) was prepared as previously described, but with the omission of potassium iodate from the mineral mix (Beckett *et al,* 1987). This basal diet mix contained less than 0.005 mg of selenium and less than 0.1 mg of iodine per kg of diet. Where appropriate, the basal diet was supplemented with 0.1 mg selenium/kg as selenite and/or 1.0 mg iodine/kg as potassium iodate. The rats were given free access to distilled water and food. The food intake was recorded daily, with the body weight of animals recorded on a weekly basis. At the end of the experiment the rats were anaesthetized with diethyl ether, and the livers and thyroids were removed and immediately frozen in liquid nitrogen. Subsequently the livers and thyroids were defrosted, homogenised in ID-I assay buffer and assayed for ID-I activity. The hepatic ID-I activity was assayed using the non-isotopic T_4 method (Section 2.05b). Thyroidal ID-I activity was assayed using both the [^{125}I]-rT_3 and the non-isotopic T_4 method (Sections 2.05a, 2.05b) to avoid possible artefacts caused by any differences in thyroid hormone concentration within the glands of the 4 groups.

6.02 : Results

a) Effect of selenium status on hepatic ID-I activity, and plasma T_3 and plasma T_4

Hepatic ID-I activity increased as the rats were supplemented with selenium (figure 6a). A maximum increase in ID-I activity of approximately 12-fold was observed when selenium supplementation reached values of 0.1 and 0.4 mg Se/kg. Selenium supplementation also caused a small rise in the plasma T_3 concentration, with a plateau observed between 0.1 and 0.4 mg Se/kg (figure 6b). In contrast, plasma T_4 concentrations were reduced by

selenium supplementation, with a decrease of approximately 60% observed at 0.1 and 0.4 mg Se/kg (figure 6b). No significant difference in food intake and body weight between the groups was found.

b) Effect of selenium deficiency on thyroidal ID-I and GPx activities

No significant difference in thyroidal ID-I activity could be seen between the selenium supplemented and selenium deficient groups (figure 6c). In contrast, GPx activity was reduced by approximately 60% in the selenium deficient group (figure 6c).

c) Effect of selenium, iodine and combined selenium/iodine deficiency on ID-I

Hepatic ID-I activity decreased by approximately 8-fold in both the selenium deficient and combined selenium/iodine deficient groups (figure 6d). Iodine deficiency alone had no significant effect of hepatic ID-I activity when compared to the selenium/iodine supplemented control group. Both the non-isotopic T_4 and the [^{125}I]-rT_3 methods essentially gave the same results with respect to the observed changes in thyroid ID-I activity (figures 6e & 6f). The specific activities of thyroidal ID-I assayed using both methods remained unchanged in the iodine deficient and combined selenium/iodine deficiency groups (figure 6e & 6f). However, selenium deficiency caused a 50% decrease in the specific activity when compared to the supplemented control (figure 6e & 6f).

Total ID-I activity within the thyroid remained unchanged in selenium deficiency, when compared to the supplemented control. In contrast, both the iodine deficient and combined deficient groups exhibited increases in total thyroidal ID-I activity (approximately a 3-fold, and a 3.7-fold increase respectively) when compared to the supplemented control. No significant difference in food intake and body weight between the groups was found.

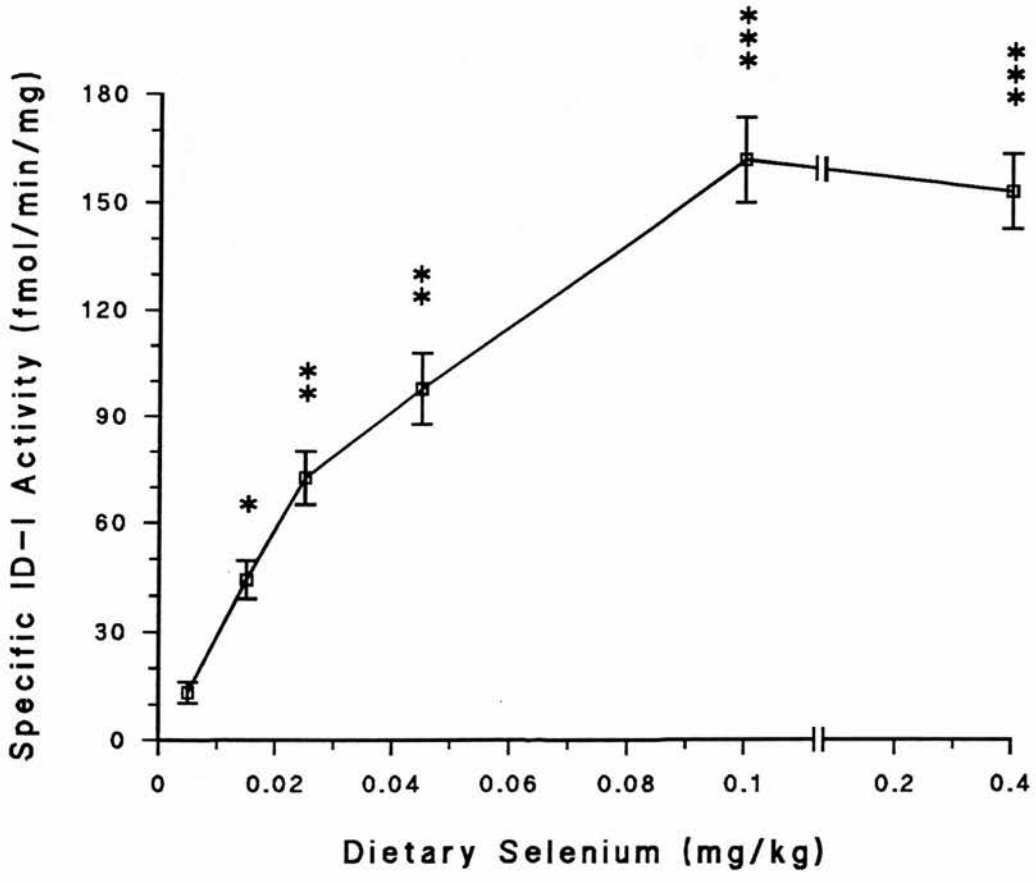
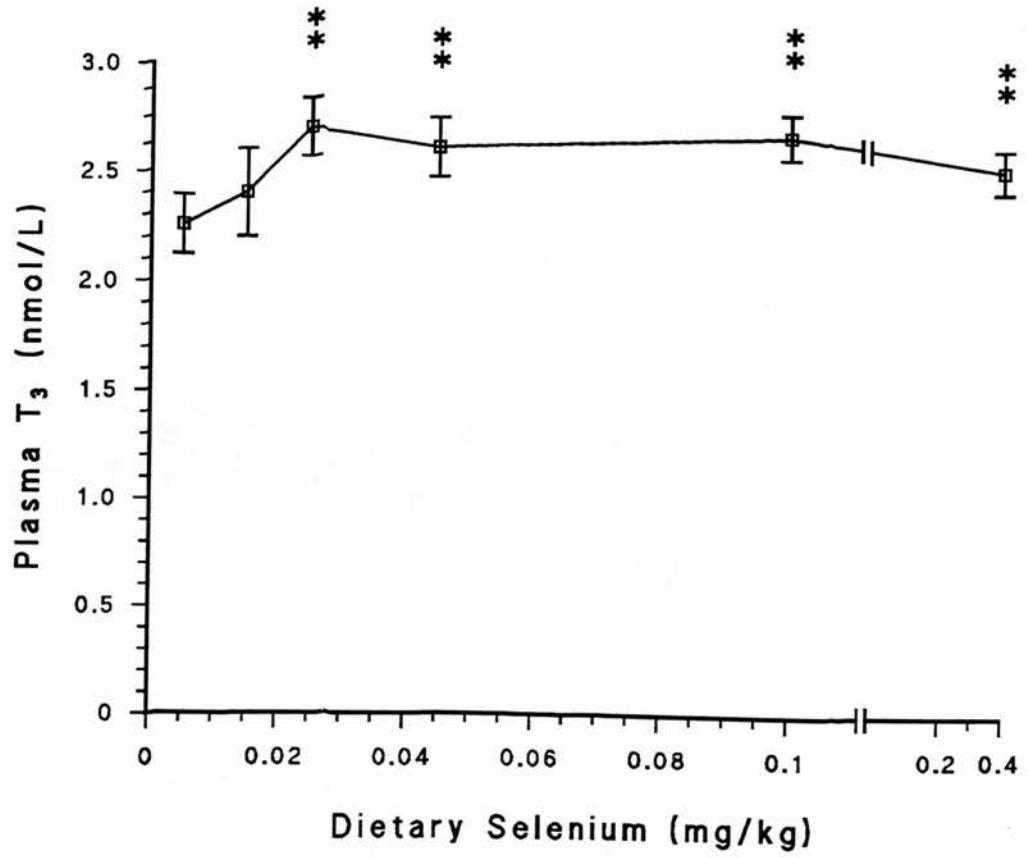


Figure 6a : Effect of dietary selenium on hepatic ID-I activity of rats maintained for 6 weeks on diets with the stated amounts of selenium supplementation. The values shown are the mean ± SEM from the livers of the various dietary groups (six rats per group). Significant increases in hepatic ID-I activity were observed above 0.015 mg Se/kg.

Figure 6b : Effect of dietary selenium on plasma T_3 (□) and T_4 (Δ) of rats maintained for 6 weeks on diets with stated amounts of selenium supplementation. The values shown are the mean ± SEM for the different dietary groups (6 animals per group). Significant increases in plasma T_3 were observed at selenium doses in excess of 0.025mg Se/kg, with significant decreases in T_4 observed at 0.015mg Se/Kg or above (compared to basal diet of 0.05 mg per kg of diet).


Plasma T_3 (nmol/L)
3.0
2.5
2.0
1.5
1.0
0.5
0
0
0.02
0.04
0.06
0.08
0.1
0.2
0.4
Dietary Selenium (mg/kg)

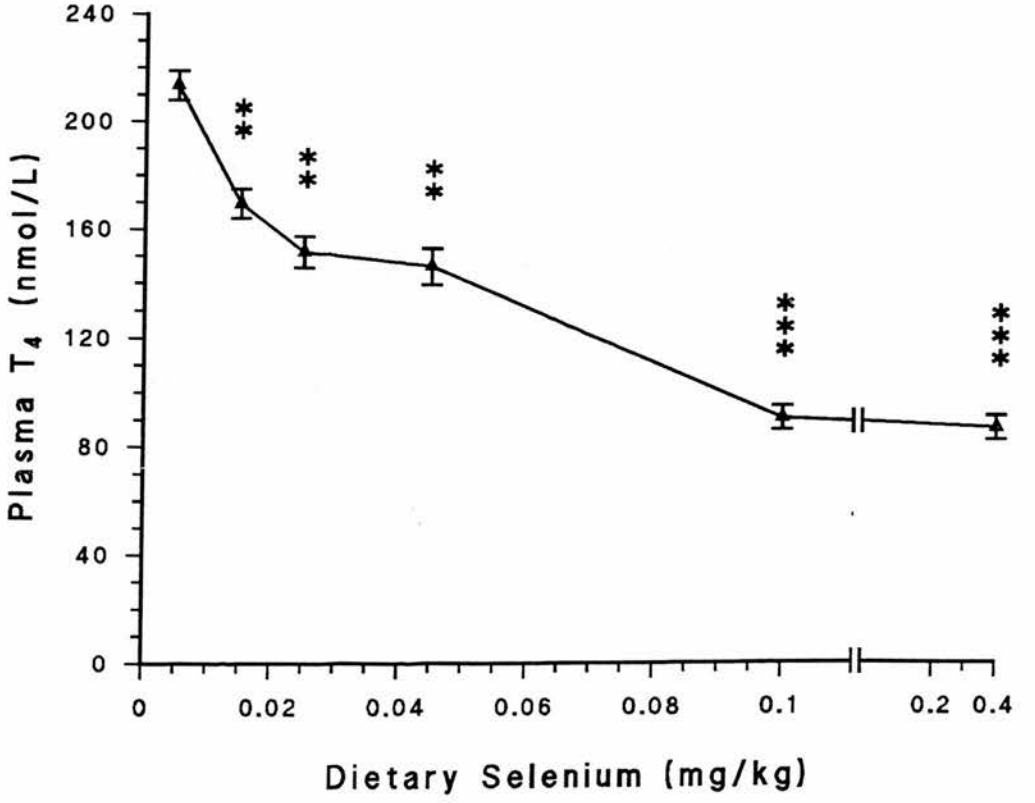
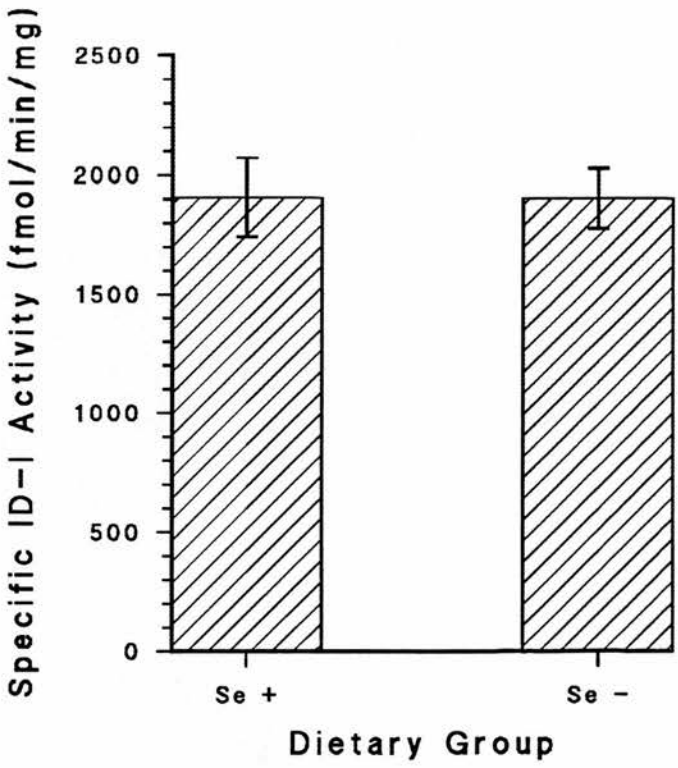
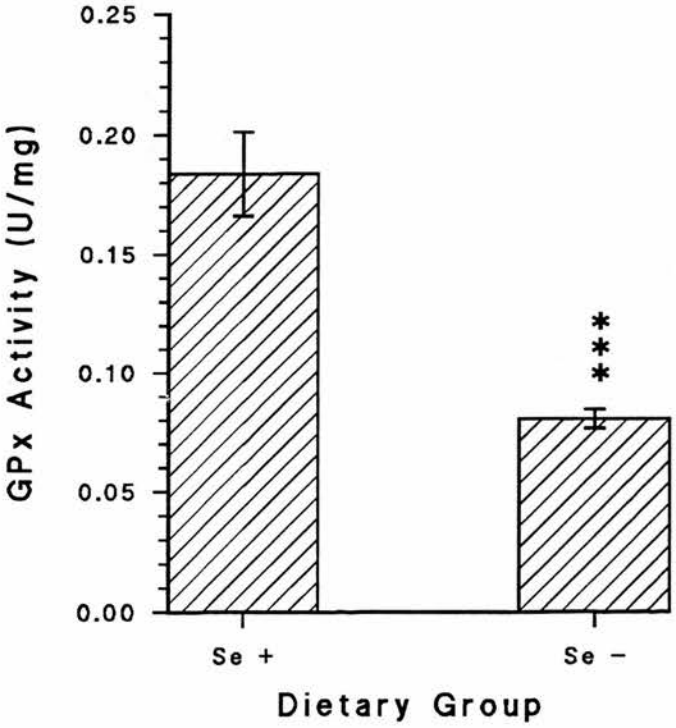

Plasma T_4 (nmol/L)
240
200
160
120
80
40
0
0
0.02
0.04
0.06
0.08
0.1
0.2
0.4
Dietary Selenium (mg/kg)

Figure 6c : Effect of selenium status on thyroidal ID-I and GPx activities measured in rats maintained on selenium supplemented (Se+) and selenium deficient (Se-) diets for 6 weeks. The values shown are the mean ± SEM for each group of rats (16 animals per group). No significant decrease in specific ID-I activity could be seen with selenium deficiency, while a significant decrease in GPx activity was seen with selenium deficiency ($p < 0.001$).


Specific ID-I Activity (fmol/min/mg)
2500
2000
1500
1000
500
0
Se +
Se -
Dietary Group


GPx Activity (U/mg)
0.25
0.20
0.15
0.10
0.05
0.00
***
Se +
Se -
Dietary Group

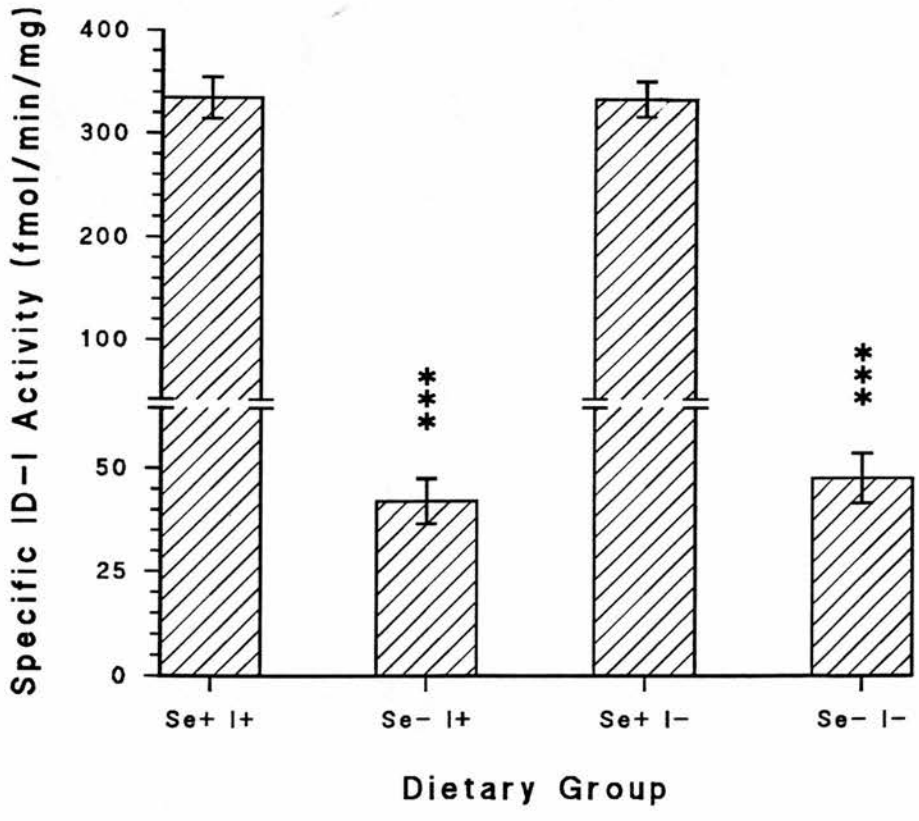
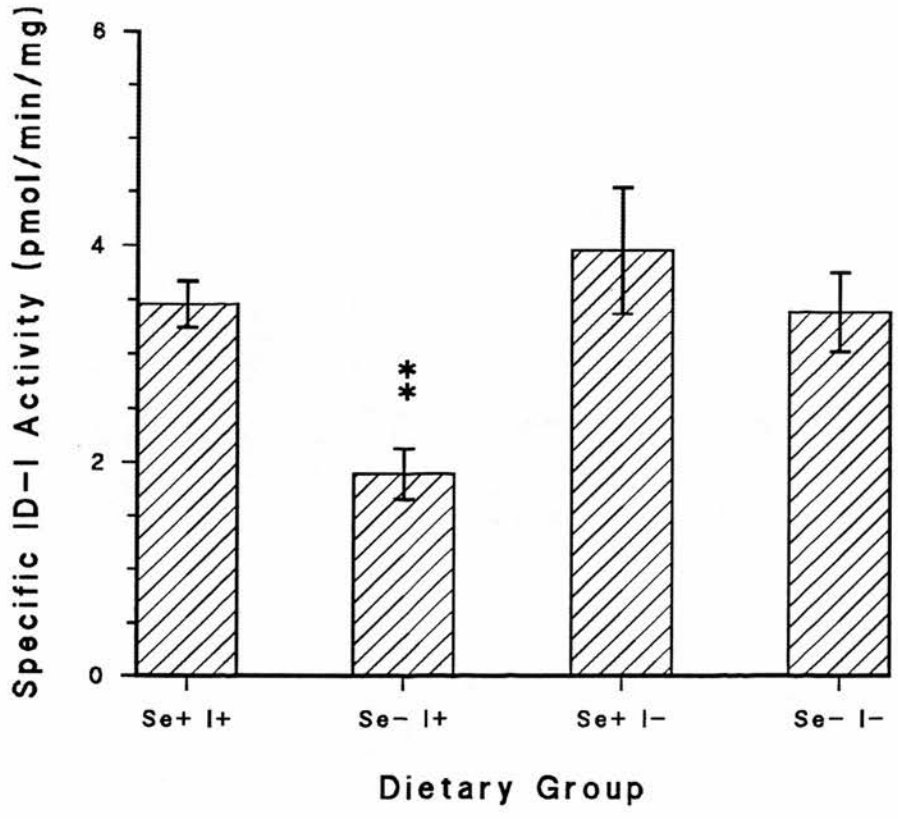


Figure 6d : Effect of selenium and iodine status on hepatic ID-I activity measured using the non-isotopic T_4 method in rats maintained for 6 weeks on one of four diets : i) selenium and iodine supplemented (Se+ I+), ii) selenium deficient (Se- I+), iii) iodine deficient (Se+ I-) or iv) combined selenium/iodine deficient (Se- I-). The values shown are the mean ± SEM of the various groups (5 animals per group). Significant decreases in hepatic ID-I activity were observed with only the Se- I+ and Se- I- groups compared to the Se+ I+ control ($p < 0.001$).

Figure 6e : Effect of selenium and iodine status on thyroidal ID-I activity measured using the non-isotopic T_4 assay method in rats maintained for 6 weeks on one of four diets : i) selenium and iodine supplemented (Se+ I+), ii) selenium deficient (Se- I+), iii) iodine deficient (Se+ I-) or iv) combined selenium/iodine deficient (Se- I-). The values shown are the mean ± SEM of the various groups (5 animals per group). A significant decrease in specific thyroidal ID-I activity was observed in the Se- I+ group. Significant increases in total activity were observed in the Se+ I- and Se- I- groups.


Specific ID-I Activity (pmol/min/mg)
6
4
2
0
**
Se+ I+
Se- I+
Se+ I-
Se- I-
Dietary Group

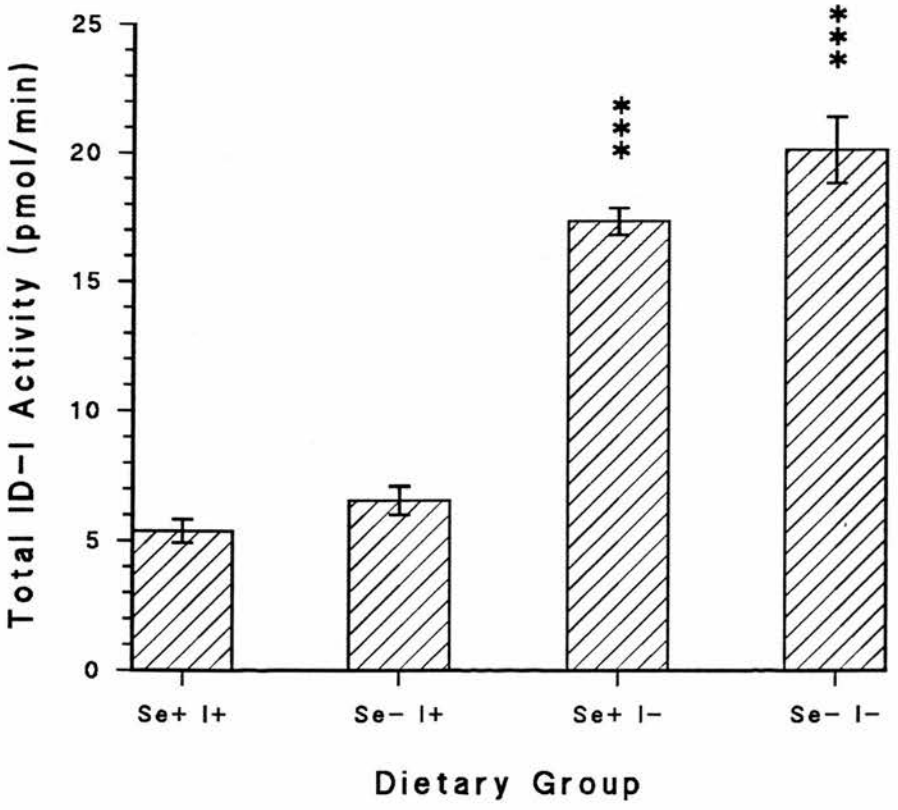
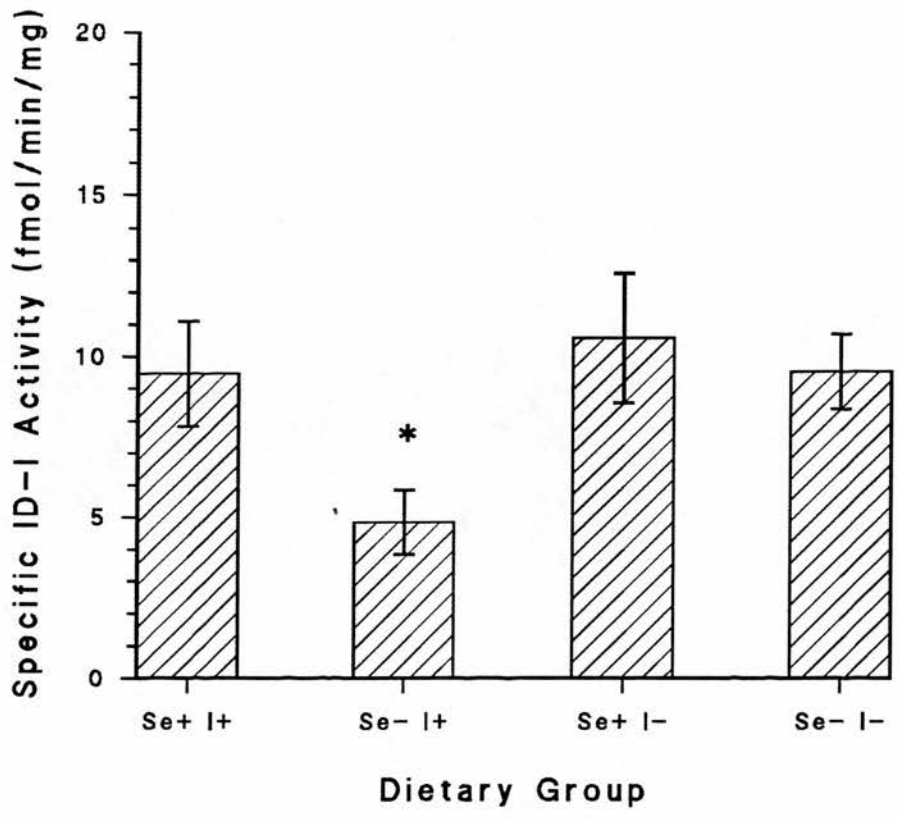
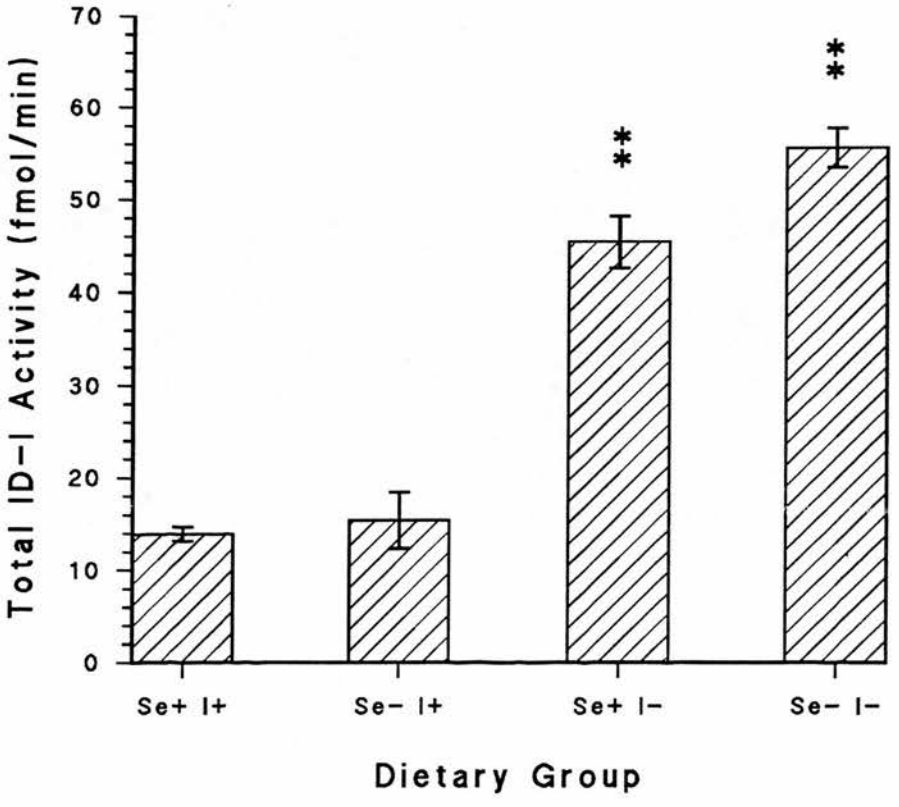

Total ID-I Activity (pmol/min)
25
20
15
10
5
0
***
***
Se+ I+
Se- I+
Se+ I-
Se- I-
Dietary Group

Figure 6f : Effect of selenium and iodine status on thyroidal ID-I activity measured using the [^{125}I]-rT_3 method in rats maintained for 6 weeks on one of four diets : i) selenium and iodine supplemented (Se+ I+), ii) selenium deficient (Se- I+), iii) iodine deficient (Se+ I-) or iv) combined selenium/iodine deficient (Se- I-). The values shown are the mean ± SEM of the various groups (5 animals per group). A significant decrease in specific thyroidal ID-I activity was observed in the Se- I+ group. Significant increases in total activity were observed in the Se+ I- and Se- I- groups.


Specific ID-I Activity (fmol/min/mg)
20
15
10
5
0
*
Se+ I+
Se- I+
Se+ I-
Se- I-
Dietary Group


Total ID-I Activity (fmol/min)
70
60
50
40
30
20
10
0
**
**
Se+ I+
Se- I+
Se+ I-
Se- I-
Dietary Group

6.03 : Discussion

The data presented in this section confirms the importance of selenium in the metabolism of thyroid hormones. Hepatic ID-I activity decreased by approximately 8-fold in selenium deficiency as compared to selenium supplemented rats (figure 6d), confirming previous work (Beckett *et al,* 1987; Beckett *et al,* 1993). Supplementation of the rats with selenite restored hepatic ID-I activity, with a plateau observed between 0.1 and 0.4 mg Se/kg of diet mix. Supplementation of selenium deficient rats with selenite also produced changes in plasma T_3 and T_4 (figure 6b). Plasma T_3 rose, while plasma T_4 decreased as selenium was fed at increasing doses to the rats. Once again a plateau was observed, corresponding to the maximum hepatic ID-I activity observed. The effect of selenium on hepatic ID-I has been reported before (Beckett *et al,* 1987) and was not unexpected as ID-I is known to be a selenoenzyme (Berry *et al,* 1991b; Mandel *et al,* 1992). As peripheral 5'-deiodination of T_4 to T_3 in tissues such as liver and kidney is known to provide approximately 80% of plasma T_3, modification of hepatic ID-I should affect plasma T_3 and T_4 concentrations (Visser, 1988). However, the observed increase in plasma T_3 as selenium was added back to the rats was much lower than might have been expected from the observed changes in hepatic ID-I activity. Reasons for this have been alluded to previously, and include an adaptive response by the thyroid, mediated by elevated plasma TSH (Beckett *et al,* 1987), and changes in the 5-deiodination of T_3 to produce T_2.

Selenium deficiency is known to increase plasma TSH, with inhibition of peripheral ID-I believed to initiate the mechanism summarised in the schematic diagram (figure 6g). Inhibition of peripheral ID-I (liver and kidney) results in a decrease in 5'-deiodination of T_4 to T_3, and hence there is an increase in plasma T_4. High plasma T_4 has been shown to cause increased degradation of pituitary ID-II (Farwell and Leonard, 1989; Farwell *et al,* 1993), and therefore there is a reduction in the level of intrapituitary T_3 production by ID-II. As the pituitary obtains most of it's T_3 from local deiodination of T_4 by ID-II, the direct

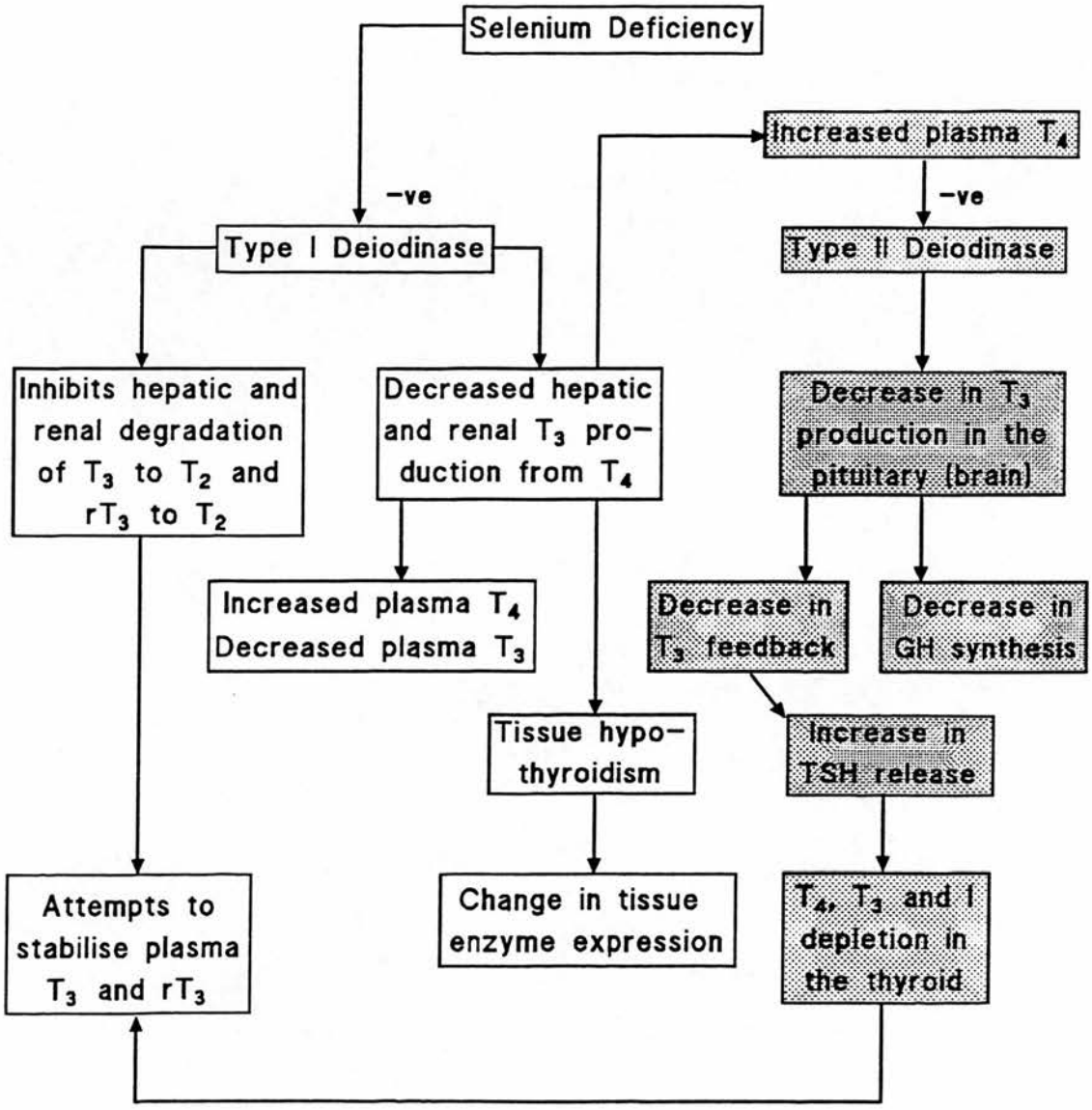


Figure 6g : Effect of selenium deficiency on ID-I, ID-II and thyroid hormone metabolism.

consequence of decreased ID-II activity is a decrease in pituitary T_3. One consequence of reduced levels of pituitary T_3 is a reduction in the negative feedback control of TSH secretion and hence increased plasma TSH levels (Arthur *et al,* 1990d). In addition, reduced pituitary T_3 also results in decreased synthesis of growth hormone. The rise in plasma TSH is known to cause preferential production of T_3 by the thyroid, though the exact mechanisms are under debate (Section 3.03). This adaptive change induced by selenium deficiency results in T_4, T_3 and I^- depletion in the thyroid, and in conjunction with a reduction in T_3 metabolism (reduced peripheral ID-I) helps to stabilise plasma T_3 (Beckett *et al,* 1993).

Selenium deficiency had only limited effects on the thyroidal ID-I activity measured, with no change or only a 50% decrease in the specific activity of thyroidal ID-I occurring (figures 6c, 6e & 6f). These changes observed in the specific activity of thyroidal ID-I were small compared to the changes observed in the level of hepatic ID-I activity which were approximately 7-fold.

Iodine and combined iodine and selenium deficient animals showed no change in the specific activity of thyroidal ID-I, but demonstrated significant increases (3 to 4-fold respectively) in the total thyroidal ID-I activity measured using either the non-isotopic T_4 or [^{125}I]-rT_3 assay method (figure 6e & 6f). These increases are most probably mediated by elevated plasma TSH and an increase in the size of the thyroid gland. Indeed, both TSH and thyroid size have been demonstrated to increase with iodine and combined iodine and selenium deficiency, with the largest increases observed in the combined deficient group (Beckett *et al,* 1993).

The expression of human thyroidal ID-I is known to be increased by TSH and hence an increase in ID-I in response to elevated TSH is not surprising provided that the supply of selenium is sufficient (Ishii *et al,* 1983). Furthermore, much larger decreases in thyroidal T_4 content were observed compared to the changes in thyroidal T_3 content, with

plasma T_3 levels maintained at the expense of plasma T_4 (Beckett *et al,* 1993; Chanoine *et al,* 1993). These observations are consistent with the reported switch to thyroidal T_3 production when plasma TSH is elevated in response to iodine deficiency (Adams and Larsen, 1973).

Selenium deficiency has been reported to exacerbate the hypothyroid effects of iodine deficiency (Beckett *et al,* 1993) and this has been implicated as a contributory factor in the pathogenesis of myxoedematous endemic cretinism (Vanderpas *et al,* 1990; Goyens *et al,* 1987). Long term selenium deficiency, in conjunction with iodine deficiency, results in increased thyroid size, elevated plasma TSH, reduced thyroidal iodine, T_3 and T_4, and a fall in plasma T_4 when compared to iodine deficiency alone (Beckett *et al,* 1993). These changes may be mediated by an further reduction in the negative feed back control of TSH secretion, when compared to iodine deficiency alone. This decrease in feed back is most probably mediated by an additional reduction in pituitary 5'-deiodination. Hence, as a result of selenium deficiency, the effects of hypothyroidism caused by iodine deficiency are exacerbated, thereby causing further impairment of growth, development and metabolism.

In addition, selenium deficiency has been postulated more recently to help protect against neurological cretinism (Corvilain *et al,* 1993). These workers proposed that decreases in peripheral ID-I (liver and kidney) and ID-II (pituitary and BAT) activity, caused by concurrent selenium deficiency, could increase plasma T_4 and hence help provide sufficient T_4 to the fetal brain to offset it's deficiency of T_4 during iodine deficiency. In addition, ID-II in rat brain has been shown to be further elevated in combined selenium and iodine deficiency, compared to iodine deficiency alone (Silva *et al,* 1984; Beckett *et al,* 1993). Hence elevated plasma T_4 and brain ID-II could reduce the effects of hypothyroidism on fetal brain development by maintaining brain T_3 levels. Corvilain and coworkers therefore put forward the hypothesis that selenium deficiency can protect

against neurological cretinism, while increasing mxyoedematous cretinism, citing Central Africa as an example. Indeed, the incidence of neurological cretinism is much lower in iodine deficient areas with concurrent selenium deficiency, in comparison to areas deficient in iodine alone, while the incidence of mxyoedematous cretinism is increased (Corvilain *et al,* 1993).

In addition to its effects on thyroid hormone metabolism, selenium deficiency caused a 60% reduction in the level of GPx found in the thyroid (figure 6c), where as a 100-fold reduction in hepatic GPx activity has been report by us to occur in rats fed the same selenium deficient diet (Beckett *et al,* 1993). This data is consistent with previously reported observations when [^{75}Se]-selenite was administered to rats *in vivo*. The brain, endocrine and reproductive tissues were found to retain ^{75}Se for far longer than liver and kidney (Behne *et al,* 1988). As a result of these findings, Behne *et al* postulated that a tissue hierarchy for selenium supply might exist. In addition to a tissue hierarchy, these workers also proposed a hierarchy at the molecular level, with a preferential supply of selenium to selenoproteins other than GPx. Indeed, one of these selenoproteins appears to be ID-I. Data present in this section clearly shows that the thyroid is supplied with selenium in preference to liver, while ID-I is supplied with selenium in preference to GPx in both the thyroid and liver.

The data presented here demonstrates that thyroidal ID-I expression can be increased *in vivo*, even in selenium deficiency, as a result of increased plasma TSH. In addition, thyroidal ID-I may have an important role in the provision of T_3 by the thyroid under hypothyroid conditions. Thyroidal GPx activity, although reduced by selenium deficiency is not as severely affected as that of the liver, confirming the findings of Behne and coworkers. In short, maintenance of thyroidal GPx activity should protect the thyroid against excessive hydrogen peroxide (H_2O_2) driven by elevated plasma TSH.

Section 7 : CONCLUDING REMARKS

In this thesis several aspects of thyroid hormone synthesis and metabolism have been studied, with particular reference to the role of thyroidal ID-I. The main objectives of the thesis were to determine the importance of thyroidal ID-I in the provision of T_3 in man and the rat, to study the dependence of this enzyme on selenium supply and to investigate the hormonal and second messenger regulation of thyroidal ID-I.

Some debate has ensued over the past few years as to the source of T_3 which accumulates in the medium bathing human thyrocytes in primary culture. Some workers have proposed that both T_3 and T_4 accumulate in the medium as a result of the secretion of preformed T_3 and T_4, with no *de novo* synthesis (Ollis *et al,* 1985). In contrast, others have published data which suggests that both T_3 and T_4 can be produced by human thyrocytes in primary culture via *de novo* synthesis (Kraiem *et al,* 1988), particularly if the thyrocytes remain differentiated (Sato *et al,* 1988). The data presented in this thesis demonstrates that human thyrocytes grown in the primary culture described (Section 2.03) do not produce T_3 or T_4 by *de novo* synthesis, even when the thyrocytes are grown in conditions which should ensure that they are differentiated. Furthermore, the data clearly shows that 5'-deiodination of endogenous T_4 is responsible for a significant proportion of T_3 which accumulates in the medium under the conditions used. In contrast, ovine thyrocytes when grown in identical conditions to the human thyrocytes produce T_3 and T_4 by *de novo* synthesis, with no evidence of T_3 production via 5'-deiodination of T_4. Indeed, the data presented in Section 4 shows that sheep and many other species lack expression of thyroidal ID-I. Why sheep, but not human thyrocytes produce T_3 and T_4 by *de novo* synthesis is unclear, though the human thyrocytes may require one or more paracrine factors in addition to those required by ovine thyrocytes.

The importance of *de novo* synthesis and 5'-deiodination in the production of T_3 by human and rat thyrocytes *in vivo* has also been open to debate, particularly in conditions which lead to increased thyroidal T_3 production. Adams and Larsen proposed that preferential *de novo* synthesis of T_3 occurred in iodine deficiency, driven by elevated plasma TSH (Adams and Larsen, 1973). Others have proposed that thyroidal 5'-deiodination of T_4 in normal and hypothyroid conditions (e.g. iodine deficient) may be important (Green, 1978; Laurberg, 1980; Laurberg, 1984; Pazos-Moura *et al,* 1991). The *in vitro* and *in vivo* data presented here supports the view that thyroidal ID-I can contribute significantly to the T_3 produced by the thyroid of man and the rat. Certain other species including sheep, lack thyroidal ID-I expression and consequently thyroidal 5'-deiodination of T_4 cannot contribute to thyroidal T_3 production in these species. A greater reliance upon thyroidal *de novo* synthesis of T_3 and peripheral production of T_3 must occur in species lacking thyroidal ID-I.

Stimulation of the TSH receptor by TSH or TSIs has been previously reported to stimulate ID-I expression (Erickson *et al,* 1982; Wu, 1983; Ishii *et al,* 1981; Ishii *et al,* 1983), with this stimulation believed to be mediated by activation of the cAMP second messenger cascade (Ishii *et al,* 1983). My work confirms that thyroidal ID-I expression can be stimulated by TSH and TSIs, most probably mediated via activation of the cAMP cascade. Furthermore, I have shown using human thyrocytes in culture that activation of the Ca^{2+}-PI second messenger cascade results in decreased ID-I expression. Activation of the Ca^{2+}-PI cascade occurs at high concentrations of TSH (Laurent *et al,* 1987; Laurent *et al,* 1989; Raspe *et al,* 1991b; Maenhaut *et al,* 1990), and is consistent with the decrease in ID-I activity observed with excess TSH (Section 4). The Ca^{2+}-PI cascade can also be activated in the thyroid by ATP, TRH and bradykinnin (Raspe *et al,* 1991b), and it is intriguing to speculate that thyroidal ID-I expression may be under the control of these other agents in addition to TSH.

Differences in tissue and protein retention of ^{75}Se has recently been demonstrated by Behne *et al*, who also postulated that a hierarchy of selenium supply to various tissues and to proteins within these tissues might occur (Behne *et al,* 1988). My work supports the existence of a tissue and molecular hierarchy for selenium supply by demonstrating preferential selenium supply to the thyroid at the expense of the liver, and within tissues to ID-I at the expense of GPx. The mechanism whereby selenium is preferentially supplied to the thyroid is unclear, however data presented here suggests the possible existence of an energy-dependent mechanism for the accumulation of selenium within the thyrocyte.

To conclude, the experiments presented in this thesis demonstrate that :-

1) In the primary culture system used here, human thyrocytes produce T_3 via 5'-deiodination of endogenous T_4, with little or no *de novo* synthesis. In contrast ovine thyrocytes lack ID-I and produce both T_3 and T_4 by *de novo* synthesis.
2) Thyroidal ID-I where present, can play a significant role in the production of T_3 in both *in vitro* and *in vivo* systems, particularly when the TSH concentration is elevated.
3) There is a great deal of species variation in the expression of thyroidal ID-I, with herbivores lacking significant levels of expression compared to omnivores.
4) Expression of thyroidal ID-I is not dependent on selenium supply per se, since thyrocytes *in vitro* and *in vivo* are able to increase ID-I expression in response to TSH, even in low selenium conditions. Indeed, selenium status in the absence of TSH has no effect on thyroidal ID-I activity.
5) Retention of selenium by thyrocytes appears to occur and involving an energy-dependent process.
6) Expression of thyroidal ID-I is under the control of the cAMP cascade which increases the expression of ID-I, while the Ca^{2+}-PI cascade appears to decrease

the expression of ID-I. As the PI pathway is stimulated by a number of neurochemicals, there may be a physiological role for the PI pathway in the regulation of ID-I expression *in vivo*.

7) TSH and TSIs can increase expression of thyroidal ID-I, presumably via the cAMP cascade. In addition, high levels of TSH can cause reduced thyroidal ID-I expression, most probably via the Ca^{2+}-PI cascade

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Section 9 : PUBLICATIONS ARISING FROM THIS THESIS

BEECH, S.G., WALKER, S.W., DORRANCE, A.M., ARTHUR, J.R., NICOL, F., LEE, D. & BECKETT, G.J. (1993). The role of thyroidal type-I iodothyronine deiodinase in tri-iodothyronine production by human and sheep thyrocytes in primary culture. *J.Endocrinol.* **132** : p 361.

BEECH, S., WALKER, S.W., ARTHUR, J.R., NICOL, F. & BECKETT, G.J. (1993). Selenium status and thyroidal iodothyronine deiodinase activity in rat and human thyrocytes. In: *Trace elements in man and animals-8*, edited by Anke, M., Meissner, D. & Mills, C.F., Gersdof : Verlag Media Touristik, p 1062.

BECKETT, G.J., **BEECH, S.**, NICOL, F., WALKER, S.W. & ARTHUR, J.R. (1993). Species differences in thyroidal iodothyronine deiodinase expression and the effect of selenium deficiency on its activity. *J.Trace.Elem.Electrolytes.Health.Dis.* **7** : p 123.

ARTHUR, J.R., NICOL, F., GILL, B.A., **BEECH, S.G.**, & BECKETT, G.J. (1993). Selenium, type-I iodothyronine 5'-deiodinase activity and thyroid hormone metabolism in the rat. In: *Trace elements in man and animals-8*, edited by Anke, M., Meissner, D. & Mills, C.F., Gersdof : Verlag Media Touristik, p 613.

BECKETT, G.J., NICOL, F., RAE, P.W.H., **BEECH, S.**, GUO, Y. & ARTHUR, J.R. (1993). The effects of combined iodine and selenium deficiency on thyroid hormone metabolism in the rat. *Am.J.Clin.Nutr.* (suppl) **57** : p 240.

The role of thyroidal type-I iodothyronine deiodinase in tri-iodothyronine production by human and sheep thyrocytes in primary culture

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ABSTRACT

We have studied the origin of tri-iodothyronine (T_3) secreted by human and sheep thyrocytes in primary culture and also the expression of type-I thyroidal iodothyronine deiodinase (ID-I) in the thyroid and liver of man and various other animals. Inhibitors of ID-I reduced T_3 secretion from human but not sheep thyrocytes. In contrast, inhibitors of de-novo thyroid hormone synthesis reduced both thyroxine (T_4) and T_3 production in sheep thyrocytes, but had no effect on the T_3 secreted by human thyrocytes. Human thyrocytes did not produce T_4 under the culture conditions used, although some endogenous T_4 was present in the cells following their isolation. Although thyrotrophin (TSH) stimulated T_3 production in both human and sheep thyrocytes, iodine in the form of potassium iodide was only essential for T_3 and T_4 production by the sheep cells. Although ^{125}I from $Na^{125}I$ was incorporated into T_3 and T_4 in TSH-stimulated sheep thyrocytes, no ^{125}I incorporation into T_3 or T_4 was detected in TSH-stimulated human thyrocytes. Using activity measurements and affinity labelling, ID-I was present in the livers of all species studied, but ID-I could not be detected in thyroid tissue from cattle, pigs, sheep, goats, rabbits, deer or llamas. In contrast, thyroid tissue from man, mice, guinea-pigs and rats had significant ID-I activity and expressed an affinity-labelled protein with a molecular mass of approximately 28·1 kDa on SDS-PAGE.

These data show that under the culture conditions used, sheep thyrocytes produced T_3 by de-novo synthesis, whilst human thyrocytes produced T_3 by deiodination of endogenous T_4. We conclude that thyroidal ID-I shows marked species difference in its expression and that, in those species which express the enzyme (man, mice, guinea-pigs and rats, in this study), it appears that it may make an important contribution to thyroidal T_3 production.

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INTRODUCTION

The thyroid gland is the exclusive source of thyroxine (T_4), while the active thyroid hormone, 3,3′,5-tri-iodothyronine (T_3), may arise from two sources. Approximately 80% of the plasma T_3 pool is produced by 5′-monodeiodination of T_4 in non-thyroidal tissue, particularly the liver and kidneys (Visser, 1988). These tissues contain a type-I iodothyronine deiodinase (ID-I) responsible for 5′-deiodination; this enzyme has been shown to be a selenoenzyme (Arthur *et al.* 1990) containing stoichiometric amounts of selenium (Behne *et al.* 1990). Cloning of the enzyme has confirmed the presence of selenium, in the form of a selenocysteine residue at the active site, coded for by a UGA triplet (Berry *et al.* 1991). ID-I activity can be inhibited by a number of compounds including propylthiouracil (PTU) and iopanoic acid (IPA), but is unaffected by methimazole, an inhibitor of thyroid hormone synthesis. All species studied to date appear to express ID-I in the liver although the enzyme may show species differences in molecular mass (Schoenmakers *et al.* 1992).

Under normal circumstances, approximately 20% of plasma T_3 arises from the thyroid, but in conditions where plasma thyrotrophin (TSH) is elevated, such as in iodine deficiency, the thyroid becomes the major source of plasma T_3 (Adams & Larsen, 1973).

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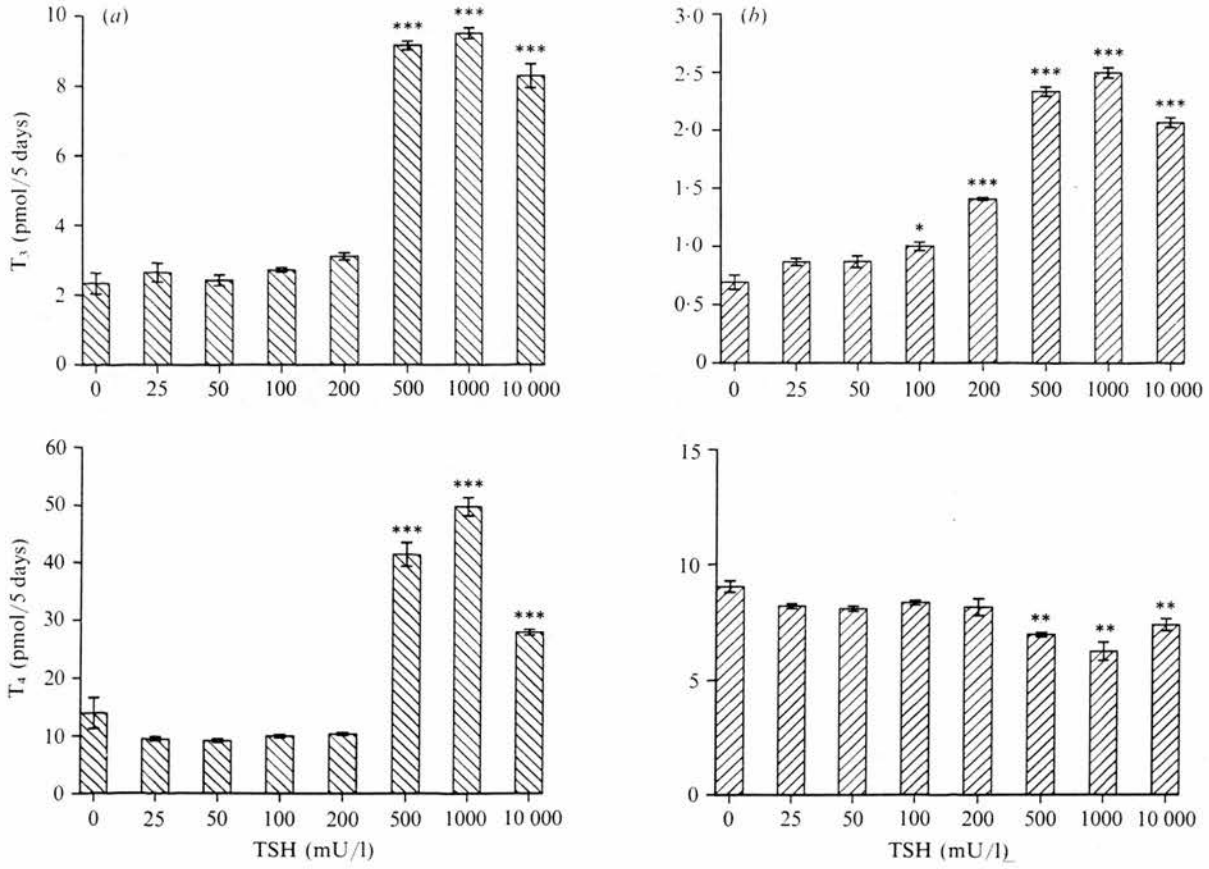

FIGURE 1. Effect of increasing doses of thyrotrophin (TSH) on thyroid hormone production from (*a*) sheep and (*b*) human thyrocytes grown in primary culture for 5 days in the presence of 10 μmol potassium iodide/l. The accumulated medium content of tri-iodothyronine (T_3) and thyroxine (T_4) was measured and the data were expressed as means ± S.E.M. of triplicate wells in a representative experiment carried out on three occasions. $^{*}P<0{\cdot}05$, $^{**}P<0{\cdot}01$ and $^{***}P<0{\cdot}001$ compared with control levels (no TSH) (Student's *t*-test).

Thyroidal T_3 is thought to arise from de-novo synthesis of and subsequent release from thyroglobulin (Visser, 1988). However, thyroids from man, dog and rat contain an ID-I that is responsive to TSH (Erickson *et al.* 1982; Wu, 1983; Pazos-Moura *et al.* 1991). When TSH is increased, the expression of ID-I is increased and it is possible that intrathyroidal deiodination of T_4 by ID-I may be an important source of thyroidal T_3 under these conditions.

In primary culture, human thyrocytes appear to produce T_3, and it has been suggested that the T_3 secreted by cultured human thyrocytes arises from de-novo synthesis (Kraiem *et al.* 1988), particularly if a follicular preparation is used and dimethyl sulphoxide (DMSO) is present (Sato *et al.* 1988). Other workers have suggested that the T_3 arises from release of preformed intracellular T_3 (Ollis *et al.* 1985). In contrast to human cultured thyrocytes, sheep thyrocytes appear to produce both T_3 and T_4 (Becks *et al.* 1987).

In this study, inhibitors of thyroid hormone synthesis (PTU and methimazole) and of ID-I (IPA and PTU) have been used to investigate T_3 production by human and sheep thyrocytes grown in primary culture and, in particular, to investigate the possibility that thyroidal deiodination of T_4 may be an important source of T_3 production. We have also studied ^{125}I incorporation into thyroid hormones and determined ID expression in liver and thyroid of various species.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's media (DMEM) and Earle's balanced salt solution (EBSS) were obtained from ICN Flow (Costa Mesa, CA, U.S.A.). Collagenase was purchased from Worthington Biochemicals Corporation via Lorne Laboratories (Twyford, Berks, U.K.), and the dispase supplied by Boerhinger Mannheim U.K. (Lewes, E. Sussex, U.K.). TSH (NIBSC code: 53/011) was supplied by the National

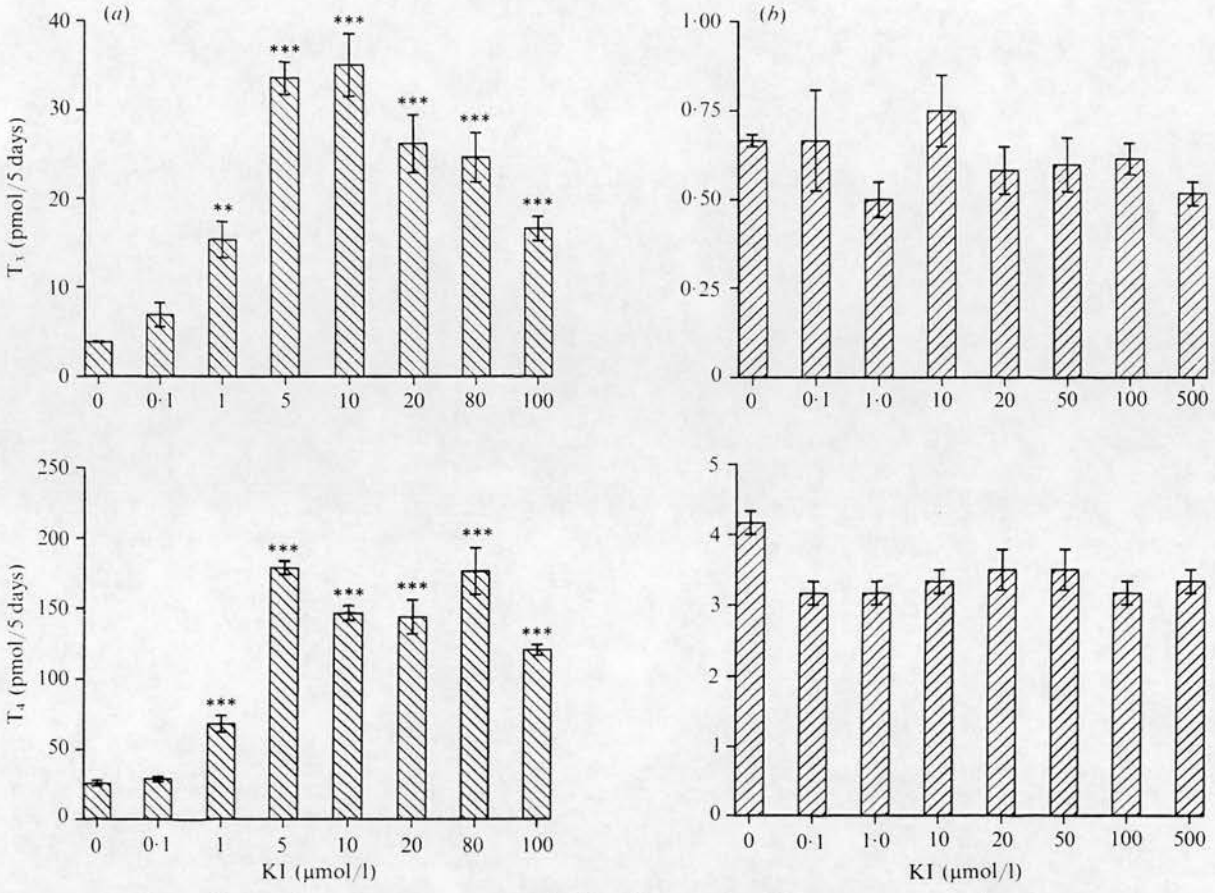

FIGURE 2. Effect of increasing doses of potassium iodide (KI) on thyroid hormone production from (*a*) sheep and (*b*) human thyrocytes grown in primary culture for 5 days in the presence of 1 mU thyrotrophin/ml. The accumulated medium content of tri-iodothyronine (T_3) and thyroxine (T_4) was measured and the data were expressed as means ± S.E.M. of triplicate wells in a representative experiment carried out on three occasions. $^{**}P<0{\cdot}01$ and $^{***}P<0{\cdot}001$ compared with control levels (no KI) (Student's *t*-test).

Institute for Biological Standards and Controls (London, U.K.) and the cellulose-F thin-layer chromatography (TLC) (5565) plates were purchased from Merck via BDH (Poole, Dorset, U.K.). Penicillin/streptomycin, amphotericin and glutamine were obtained from NBL (Cramlington, Northumberland, U.K.). ^{125}I-Labelled T_3, T_4, reverse tri-iodothyronine (rT_3) and $Na^{125}I$ were obtained from Amersham International plc, Amersham, Bucks, U.K. Anti-T_3 and T_4 were kindly supplied by the Scottish Antibody Production Unit, Carluke, Lanarkshire, U.K. All other reagents including control processed serum replacement 5 (CPSR-5) were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.).

Isolation and culture of human and sheep thyrocytes

Human thyrocytes were isolated from normal and colloid goitre tissue (surplus to routine pathological requirements) from patients undergoing thyroid surgery. The cells were isolated using a modified version of the dog thyroid cell culture method of Rapoport (1976). The tissue was sliced using a Stadie–Riggs microtome and the slices were finely minced with scissors. The resulting fragments were then washed four times with EBSS and digested for 2 h in 50 ml of an enzyme cocktail containing dispase (5 mg/ml), trypsin (0·25%, w/v) and collagenase (1 mg/ml) in EBSS. Following digestion, an equal volume of 2% (w/v) bovine serum albumin (BSA) in EBSS was added and the mixture filtered through a 100 μm mesh gauze to remove undigested tissue. The resulting filtrate, containing released thyroid cells, was centrifuged at 125 ***g*** for 15 min to pellet the thyrocytes and leave the majority of erythrocytes in suspension. The pellet was then resuspended in EBSS, re-centrifuged twice and the cells finally resuspended in 50 ml DMEM containing 10% (v/v) CPSR-5 (fetal calf serum treated to

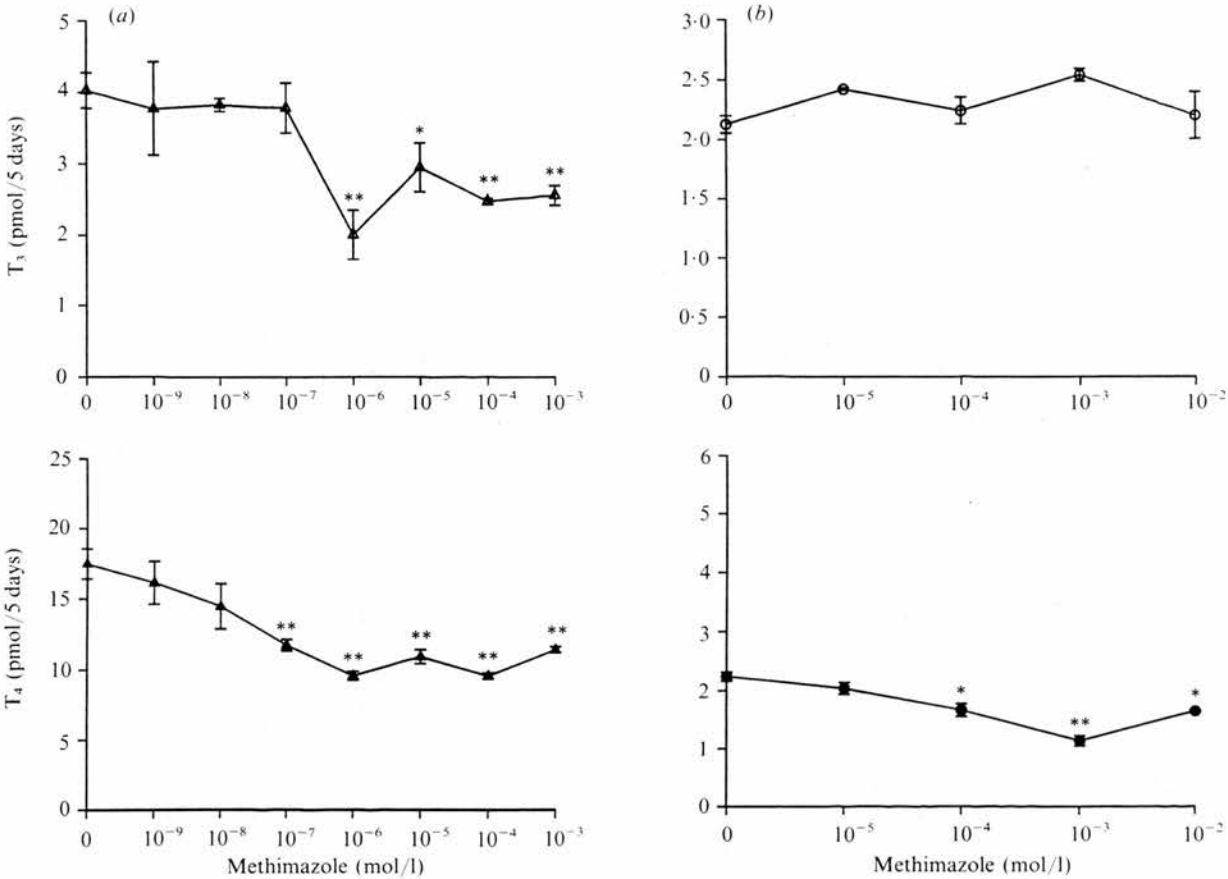

FIGURE 3. Effect of 5 days exposure to methimazole on thyroid hormone production from (*a*) sheep and (*b*) human thyrocytes grown in primary culture. The accumulated medium content of tri-iodothyronine (T_3) (○, human; △, sheep) and thyroxine (T_4) (●, human; ▲, sheep) was measured and data were expressed as means ± S.E.M. of triplicate wells in a representative experiment carried out on three occasions. $^*P<0.05$ $^{**}P<0.01$ compared with control levels (no methimazole) (Student's *t*-test).

emove immunoglobulins and endotoxins), penicillin 100 U/ml), streptomycin (100 μg/ml), amphotericin 3 (2·5 μg/ml) and added glutamine (2 mmol/l). The ell suspension was then filtered through a 30 μm nesh gauze and the cell yield measured with a modi- ed Neubauer haemocytometer. Finally the thyro- ytes were plated out in DMEM/10% CPSR-5 into 2-well plates (2·5 cm diameter) at a density of 5×10^5 ells/well in 1 ml medium. The cells were incubated t 37 °C in an atmosphere of 5% CO_2 for 24 h before ny additions were made.

Sheep thyrocytes were isolated, using the same nethod, from sheep thyroid tissue obtained within 0 min of death from animals in the local abattoir.

ffect of TSH and KI concentration on T_3 and T_4 oduction by human and sheep thyrocytes

fter 24 h, the medium was removed from the human nd sheep thyrocytes in monolayer culture and the cells were washed with 1 ml EBSS before fresh medium containing a fixed dose of KI (10 μmol/l) was added. Wells (in triplicate) contained a range of TSH doses. In other experiments, the TSH was maintained at 1 mU/ml whilst the amount of KI added to the medium was varied. The concentration of T_3 and T_4 in the culture medium was then determined by in-house radioimmunoassay after a further 5 days in culture.

Effect of IPA, PTU and methimazole on T_3 and T_4 production from human and sheep thyrocytes

After 24 h of incubation, the medium from both the human and sheep thyrocytes was removed and the cells were washed with EBSS before fresh medium was added containing TSH (1 mU/ml), KI (10 μmol/l) and inhibitors, as appropriate. The inhibitors used were increasing doses of IPA (an ID-I

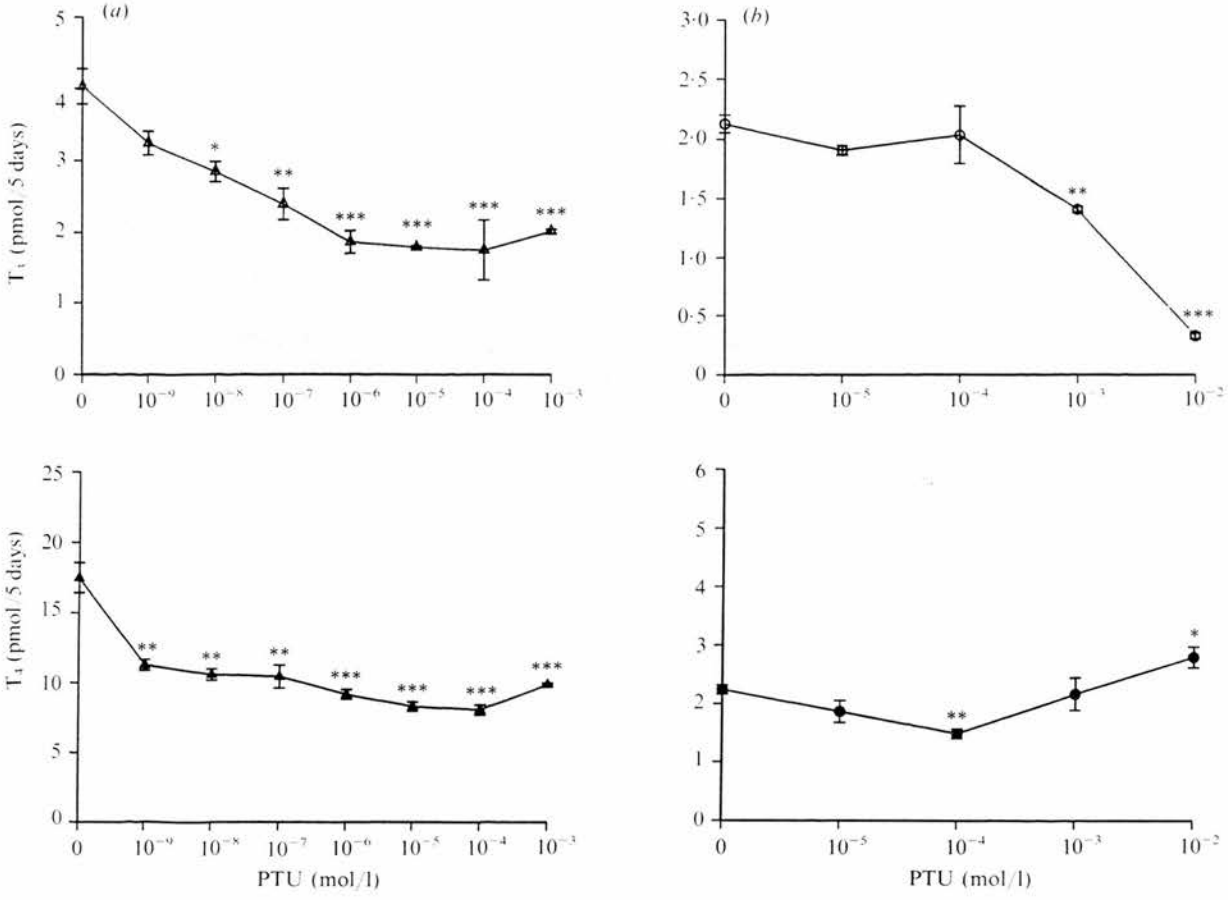

FIGURE 4. The effect of 5 days exposure to propylthiouracil (PTU) on thyroid hormone production from (*a*) sheep and (*b*) human thyrocytes grown in primary culture. The accumulated medium content of tri-iodothyronine (T_3) (○, human; △, sheep) and thyroxine (T_4) (●, human; ▲, sheep) was measured and data were expressed as means ± S.E.M. of triplicate wells in a representative experiment carried out on three occasions. $^*P<0{\cdot}05$, $^{**}P<0{\cdot}01$ and $^{***}P<0{\cdot}001$ compared with control levels (no PTU) (Student's *t*-test).

inhibitor), methimazole (inhibitor of T_3 and T_4 de-novo synthesis) or PTU (inhibitor of both ID-I and de-novo synthesis). Thyroid hormone production after a further 5 days was quantified by radioimmunoassay of the T_3 and T_4 accumulated in the culture media.

Previous experiments had shown that, within an experiment, the addition of TSH, KI, IPA, PTU and methimazole produced no significant changes in protein content of the cells during the 5 days of culture. Results of T_3 and T_4 production have therefore been expressed as the total amount of T_3 and T_4 produced in 5 days by each incubation and not corrected for protein.

Incorporation of ^{125}I into T_3 and T_4 by human and sheep thyrocytes

Human and sheep thyrocytes were plated out in 75 cm^2 flasks in 10 ml DMEM/10% CPSR-5 at a density of 10 million per flask. After 24 h, the medium was removed, and the cells were washed with EBSS before fresh medium was added containing 50 μCi $Na^{125}I$ (specific activity > 1200 μCi/μg) with or without TSH (1 mU/ml). After a further 5 days of incubation the medium was removed and T_3 and T_4 were immunoprecipitated by incubation for 15 h at 25 °C with solid-phase anti-T_3/T_4 immunoglobulins added at amounts sufficient to precipitate more than 95% of T_3 and T_4 produced by the cells. The samples were then centrifuged at 180 *g* for 15 min, and the antibody pellet was washed with 20 ml 1 mol KI/l and re-centrifuged. The pellet was then resuspended in 5 ml methanol and the suspension left for 15 h at 25 °C to extract the bound hormones. After further centrifugation at 180 *g* for 15 min, the methanolic supernatant was evaporated to dryness and the residue, containing the immunoextracted T_3 and T_4, taken up in 100 μl methanol. The samples were

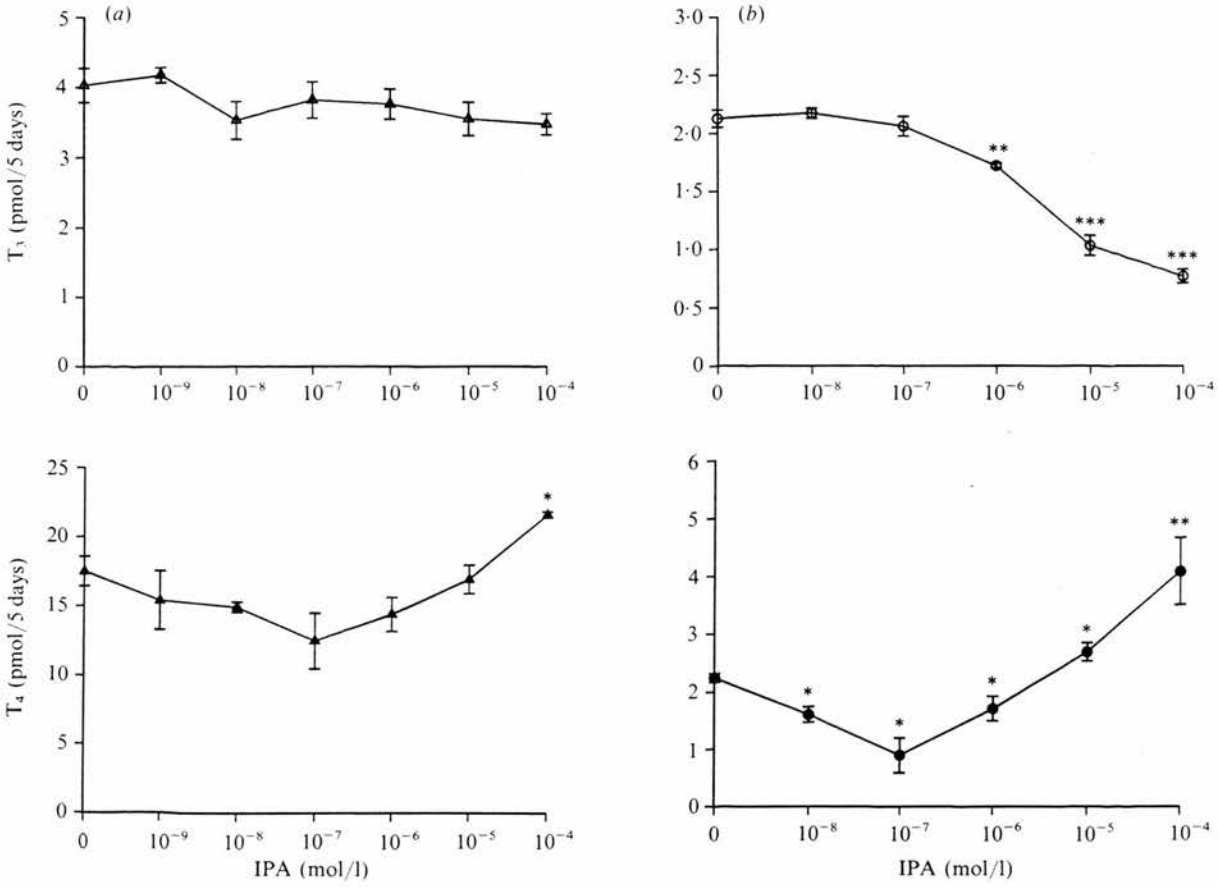

FIGURE 5. The effect of 5 days exposure to iopanoic acid (IPA) on thyroid hormone production from (*a*) sheep and (*b*) human thyrocytes grown in primary culture. The accumulated medium content of tri-iodothyronine (T_3) (○, human; △, sheep) and thyroxine (T_4) (●, human; ▲, sheep) was measured and data were expressed as means ± S.E.M. of triplicate wells in a representative experiment carried out on three occasions. $^{*}P<0{\cdot}05$, $^{**}P<0{\cdot}01$ and $^{***}P<0{\cdot}001$ compared with control levels (no IPA) (Student's *t*-test).

then run on 20 cm^2 cellulose/TLC plates using chloroform:tertiary butanol:ammonia (2 mol/l), (60:376:70; by vol.) as the developing solvent. Autoradiography was used to demonstrate the presence of iodinated T_3 and T_4 (48 h exposure). Appropriate ^{125}I-labelled standards of T_3 and T_4 were also run on the same plate. No detectable $Na^{125}I$ was carried over on to the TLC plates, as determined by autoradiography.

Determination of ID-I activity in thyroid liver homogenates from various species

Thyroidal and hepatic tissue from cattle, pigs, sheep, goats, rabbits, deer, llamas and rats were obtained within 30 min of death, whilst the human tissue was obtained as for culture. All tissue was stored at −70 °C before portions of these tissues were homogenized in a potassium phosphate (100 mmol/l) buffer containing 5 mmol EDTA/l and 20 mmol dithiothreitol (DTT)/l (pH 7·4) to obtain a 20% (w/v) homogenate. After centrifugation for 15 min at 300 ***g***, the supernatant was removed and ID-I activity assayed by measuring the release of ^{125}I from ^{125}I-labelled rT_3, as described previously (Sawada *et al.* 1986) but using rT_3 at a final concentration of 2 nmol/l. Under these conditions, reactions were linear up to 60 min with 25–180 μg of protein.

Affinity labelling of liver and thyroid homogenates

Liver and thyroid homogenates from the various species were labelled with N-bromoacetyl[^{125}I]rT_3 using a modified version of the method described by Nikodem *et al.* (1980). This method is outlined in our previous paper (Arthur *et al.* 1991), except that whole homogenates were used in the present study instead of microsomes. The labelled homogenates were then subjected to sodium dodecyl sulphate-polyacrylamide

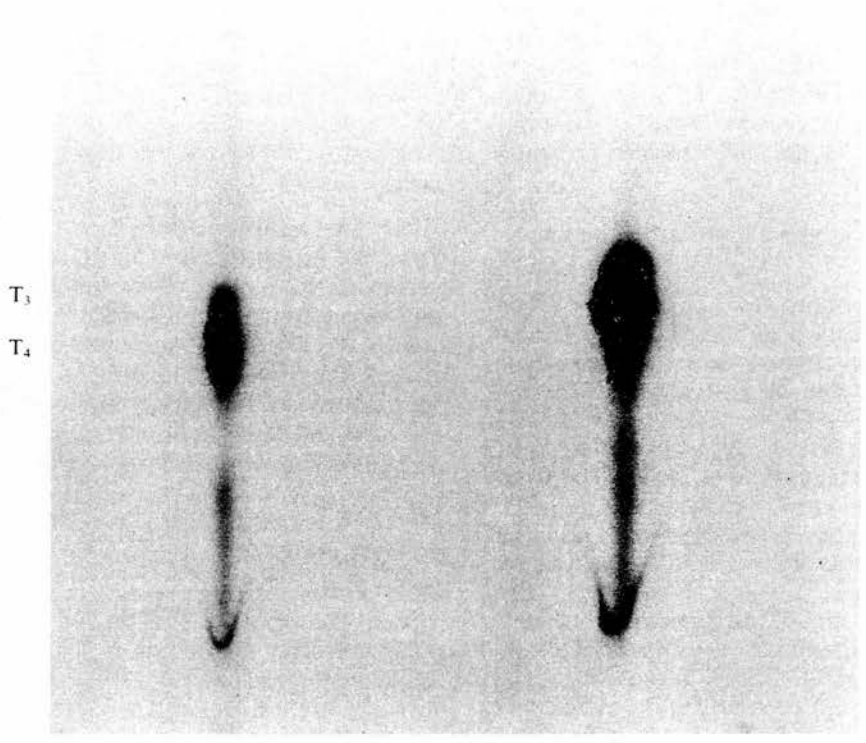

FIGURE 6. A thin-layer chromatograph of radiolabelled tri-iodothyronine (T_3) and thyroxine (T_4) immunoprecipitated from cell medium following 5 days incubation of sheep and human thyrocytes with $Na^{125}I$, showing the incorporation of ^{125}I into T_3 and T_4 in sheep but not human thyrocytes under thyrotrophin (TSH) stimulation. Lane 5 contains similar amounts of the material run in lane 2 with the addition of ^{125}I-labelled T_3 and ^{125}I-labelled T_4 to act as standards.

TABLE 1. Activities of iodothyronine deiodinase type-I in the thyroids and livers of various species, expressed as the amount of iodine released from reverse tri-iodothyronine/min per mg protein

	Thyroidal activity (fmol I/min per mg)	**Hepatic activity** (fmol I/min per mg)
Species		
Rat	1296	1045
Guinea-pig	283	—
Mouse	79	—
Human	56	437
Goat	3·6	1052
Cattle	1·9	1066
Rabbit	0·9	—
Sheep	0·4	170
Pig	0·4	291
Llama	0·3	119
Deer	ND	—

ND, not detectable; —, not determined.

gel electrophoresis (SDS-PAGE) with molecular mass markers and affinity-labelled proteins visualized using autoradiography.

RESULTS

Effect of TSH and KI concentration on T_3 and T_4 secreted by human and sheep thyrocytes

T_3 and T_4 production by sheep thyrocytes showed clear dose-dependence on both TSH and KI (Figs 1*a* and 2*a*). The optimal concentration for TSH was 1 mU/ml. In the case of KI, T_3 and T_4 production rose to a plateau in some experiments, but decreased with levels of KI beyond 10 μmol/l in others. Accordingly, 10 μmol/l was used as the optimum KI concentration. In human thyrocytes T_4 production was low and failed to be stimulated by TSH or iodide. In fact,

at a dose of TSH of 1 mU/ml, T_4 in the media was significantly reduced (Fig. 1*b*). Although T_3 production was stimulated by TSH (optimum 1 mU/ml), no effect of KI addition could be seen (Fig. 2*b*). Human thyrocytes secreted approximately 25% of the amount of T_3 and T_4 secreted by the sheep thyrocytes when under TSH stimulation.

Effect of inhibitors on T_3 and T_4 production by human and sheep thyrocytes

In sheep thyrocytes, methimazole and PTU produced a dose-dependent inhibition of both T_3 and T_4 accumulation, with a maximal effect for both compounds at 1 mmol/l (Figs 3*a* and 4*a* respectively). At this dose, methimazole caused 35% decreases in T_3 and T_4, while PTU caused 50% decreases in both T_3 and T_4. IPA had no significant effect on T_3 and T_4 accumulation (Fig. 5*a*).

In human thyrocytes, methimazole caused no large changes in T_3 and T_4 secretion (Fig. 3*b*). In contrast to the sheep thyrocytes, PTU and IPA caused a significant increase in T_4 and a corresponding decrease in T_3 accumulation (Figs 4*b* and 5*b*). Maximal effects occurred at 10 mmol PTU/l which caused a 50% increase in T_4 and an 80% decrease in T_3. In the case of IPA, maximal effects occurred at 100 μmol IPA/l and consisted of an 80% increase in T_4 and a 70% decrease in T_3.

Incorporation of ^{125}I into T_3 and T_4 by human and sheep thyrocytes

In the presence of TSH, incorporation of ^{125}I into T_3 and T_4 occurred in sheep thyrocytes, with no detectable incorporation occurring when TSH was absent (Fig. 6). With human thyrocytes no ^{125}I incorporation into T_3 and T_4 could be detected irrespective of the presence or absence of TSH.

ID-I activity in thyroid and liver homogenates from various species

There was a large variation in the activity of ID-I measured in the different livers, but all showed substantial levels of activity ranging from 119 to 1066 fmol/min per mg (Table 1). Only in the thyroid tissue from rat, man, mouse and guinea-pig could significant activity be demonstrated, with the rat having the highest specific activity (1296 fmol/min per mg). All other species had very low specific activities (0·3–3·6 fmol/min per mg).

Affinity labelling of thyroid and liver homogenates from various species

Liver homogenates from all species showed an ID-I band in the region of 27·3 to 29·0 kDa but there were small but clear differences in the molecular mass of this band between species. A second band was also apparent in the liver of all species which had a consistent molecular mass of 53·2 kDa. When thyroid homogenates were subjected to SDS-PAGE, rat and human exhibited a band corresponding in molecular mass to the ID-I demonstrated in the liver of the corresponding species (Fig. 7; Table 2). For thyroid tissue of the other species studied in Fig. 7, although bands were found with molecular masses in the region 30·5–33 kDa, no band was found with the lower

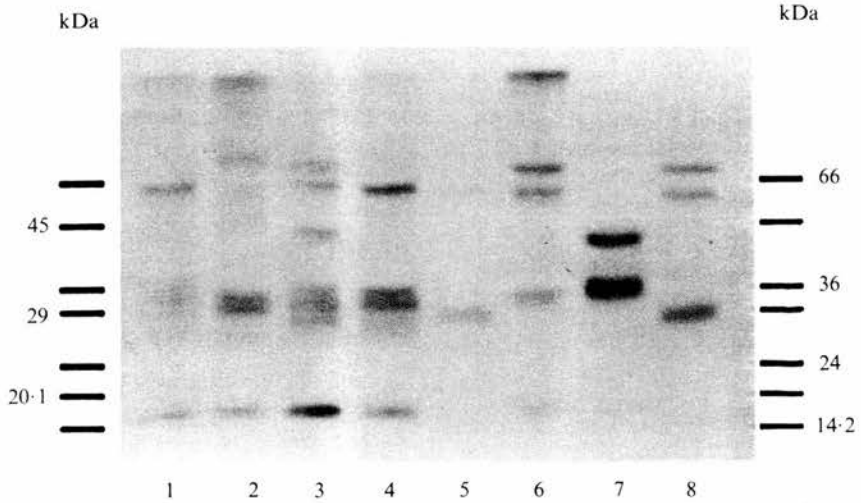

FIGURE 7. Autoradiograph of an SDS-PAGE gel of thyroid homogenates from various species labelled with bromoacetyl [^{125}I]rT_3. Tracks 1–8 show sheep, llama, human, cattle, deer, pig, goat and rat respectively. The mobility of molecular mass markers is shown.

molecular mass band corresponding to that of ID-I in the liver of the same species. The presence of the ID-I band in the thyroid of the various species thus correlated well with ID-I activity, indicating that the very low enzyme activities were due to a lack of expression of the enzyme, rather than inhibition of activity. All the thyroid glands from these species had selenium-containing glutathione peroxidase activity, confirming that the lack of ID-I activity was not due to selenium deficiency (data not shown).

TABLE 2. Molecular mass of hepatic iodothyronine deiodinase type I (ID-I) from various species as determined by autoradiography of an SDS-PAGE gel of bromoacetyl [^{125}I]rT$_3$-labelled homogenate. Using thyroid tissue, bands observed within the molecular mass region of hepatic ID-I in the corresponding species are also shown. Only rat and human thyroid tissue had a band corresponding to hepatic ID-I

	Molecular mass of affinity-labelled protein (kDa)	
	Liver	Thyroid
Species		
Rat	28·1	28·1
Human	28·1	28·1
Goat	27·3	32·9
Cattle	28·9	32·9
Sheep	27·3	30·4
Pig	28·1	31·6
Llama	28·1	30·5

DISCUSSION

In sheep thyrocytes, the production of T_3 and T_4 was dependent on TSH and KI and was inhibited by methimazole and PTU, substances which inhibit de-novo synthesis. In addition ^{125}I was incorporated into both T_3 and T_4 in the presence of TSH. These data clearly established that sheep thyrocytes were able to produce both T_3 and T_4 by de-novo synthesis under these culture conditions. In the sheep, ID-I can play no role in thyroidal T_3 production as the thyroid does not express significant levels of this enzyme in this species. In support of this, IPA (which inhibits ID-I) had no effect on T_3 production in sheep thyrocytes.

In contrast, T_3 production in human thyrocytes was inhibited by IPA or PTU, but not by methimazole. We also observed that T_3 production was independent of iodide and that no ^{125}I was incorporated into T_3 or T_4, even under TSH stimulation. These observations are consistent with T_3 production arising from deiodination of endogenous T_4 present within the thyrocytes following their isolation, with little or no T_3 being produced in a de-novo fashion. Unlike sheep thyrocytes, human thyrocytes expressed ID-I activity and therefore the TSH-dependence of T_3 production in the isolated cell system could be attributed to the TSH-responsive nature of ID-I in human thyroid tissue. High levels of TSH have been shown to stimulate the activity of the enzyme, probably in a cyclic AMP-dependent manner (Murakami *et al.* 1990).

These results obtained with the human thyrocytes (Figs 3–5) differ from those of Kraeim *et al.* (1988), who proposed that T_3 production in isolated human thyrocytes arises from de-novo synthesis. These workers based their conclusions on the observation that PTU inhibited T_3 production, an observation confirmed by our work. However, since PTU inhibits both T_3 synthesis and deiodination of T_4, the inhibitory effect of PTU cannot distinguish between these two sources of T_3. Kraeim *et al.* (1988), also reported (but did not publish any supportive data) that methimazole inhibited T_3 production, while the effects of IPA were not studied. We found no evidence of inhibition of T_3 production, even at methimazole concentrations as high as 10 mmol/l. Differences between our study and that of Kraeim *et al.* (1988) may lie in the culture system since Kraeim *et al.* (1988) used cells cyro-preserved in medium 199/10% fetal calf serum/10% DMSO with Hepes (25 mmol/l).

Sato *et al.* (1988) using a culture system of isolated human follicles were able to demonstrate de-novo synthesis of T_3 and T_4 in the presence of DMSO (1·7%) and low concentrations of fetal calf serum (1%). They postulated that synthesis occurred by DMSO preventing dedifferentiation and that high levels of fetal calf serum led to deterioration of thyroid function.

Our studies show that, although all the species investigated contained an active ID-I in their liver, the thyroidal ID-I activity showed a high degree of species variation. As previously reported (Toyoda *et al.* 1992), we found that human thyroid expresses ID-I. We have also demonstrated that ID-I is expressed in the thyroids of other omnivores (mouse, guinea-pig and rat). In contrast, the thyroids of herbivores and the pig seem to lack ID-I activity, but why this should be is unclear. In cattle, pigs, sheep, goats, rabbits, deer and llamas, thyroidal ID-I is not significantly expressed and cannot therefore provide a source of thyroidal T_3 in cases of iodine deficiency. Man, mice, guinea-pigs and rats have significant levels of thyroidal ID and hence this could prove to be a useful secondary system for maintaining plasma T_3 under conditions of hypothyroid stress, such as in iodine depletion, where increased TSH leads to stimulation of ID-I synthesis.

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SELENIUM STATUS AND THYROIDAL IODOTHYRONINE DEIODINASE ACTIVITY IN RAT AND HUMAN THYROCYTES

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Introduction

Approximately 80% of plasma 3',5',5-triiodothyronine (T_3) is derived from 5'monodeiodination of thyroxine (T_4), a reaction catalysed by the selenoenzyme type-I iodothyronine deiodinase (ID-I) (**1-3**) in non-thyroidal tissues such as liver, kidney and muscle. Cloning of the gene for the enzyme and site directed mutagenesis have confirmed the presence of a selenocysteine residue at the active site, coded for by a UGA triplet. The thyroid of some, but not all species (**4**), also express ID-I. However unlike hepatic ID-I, thyroidal ID-I can be stimulated by thyrotropin (TSH) (**5-7**). In selenium deficiency the activity of hepatic ID-I is often decreased to less than 10% of the activity found in selenium-supplemented rats and the activity of hepatic glutathione peroxidase (GPx), a selenoenzyme involved in the protection of tissue from peroxidative damage, is decreased to levels of <1.0% of activities found in selenium-supplemented animals (**8**). There is good evidence to suggest that in selenium deficiency the brain, endocrine and reproductive tissues retain selenium more efficiently than the liver, and that within a tissue there is a hierarchy for selenium supply to the various selenoproteins (**9**).

In this paper we present data on the effects of selenium deficiency on thyroidal ID-I activity in rat and man. We also show that selenium uptake and retention by cultured thyrocytes is a temperature dependent process.

Methods and Results

Effect of TSH and selenite on ID-I activity in cultured human thyrocytes

Human thyrocytes were isolated from normal and colloid goitre tissue from patients undergoing thyroid surgery using a modified version of the dog thyroid cell culture method of Rapoport (**10**) as described previously (**4**).

After 24 h, the medium was removed from the thyrocytes and the cells washed twice with 1ml Earle's balanced salt solution (EBS) before fresh Dulbecco's modified Eagle's

medium (DMEM) was added (no fetal calf serum). Selenite was added (50nmol/L or 1000nmol/L), in the presence or absence of 1U/L TSH. After a further 24 h, the medium was removed and the cells washed and treated with selenite and TSH as on the first medium change. The thyrocytes were then cultured for a further 4 days before being washed twice with EBS and detached from the plate in potassium phosphate (100mmol/L) buffer containing 1mmol/L EDTA (pH 7.4) using a rubber policeman. The thyrocyte suspensions were sonicated and the ID-I activity assayed by measuring the release of ^{125}I from ^{125}I-rT_3, as described previously (**11**).

In the absence of TSH and selenite the activity of ID-I was very low (table 1) and a small but significant increase in activity was observed when selenite was added to the culture medium. In the absence of selenite the activity of ID-I was increased 20 fold on addition of TSH. On addition of selenite a further 3 fold increase in ID-I activity was found with a plateau of ID-I activity occurring at a selenite concentration of between 50 and 1000 nmol/L. **Table 1:-**

	No Se (fmol/hr/mg)	50nmol/L Se (fmol/hr/mg)	1000nmol/L Se (fmol/hr/mg)
No TSH	22.8 ± 19.0	136.3 ± 48.2	102.3 ± 43.2
1 U/L TSH	406.7 ± 70.3	1251 ± 111	1340 ± 284

The effect of dietary selenium and iodine on the activity of ID-I and GPx in the rat

Male, Hooded Lister, weanling rats of the Rowett Institute strain were divided into 4 groups and offered one of the following diets for 10 weeks : 1) selenium and iodine supplemented control (Se+I+), 2) selenium deficient (Se-I+), 3) iodine deficient (Se+I-), and 4) selenium and iodine deficient (Se-I-). The basal diet (Se-I-) was prepared as described previously (**12**). Where appropriate, the basal diet was supplemented with 0.1 mg Se/kg as selenite, and/or 1.0mg I/kg as potassium iodate. Distilled water and food were available ad libitum. After 10 weeks the liver and thyroids of the animals were removed into liquid nitrogen and kept at -70°C prior to assay of ID-I and GPx activities in tissue homogenates. (**11, 13**).

In both groups of Se- animals, thyroidal GPx activity was decreased to approximately 50% of the activity found in the Se+ groups; in the livers of the same animals GPx activity was reduced by selenium deficiency to approximately 1% of the control values. Hepatic ID-I activity was reduced by selenium deficiency to 10% of control values but thyroidal ID-I increased in both the iodine and selenium deficient groups. (table 2).

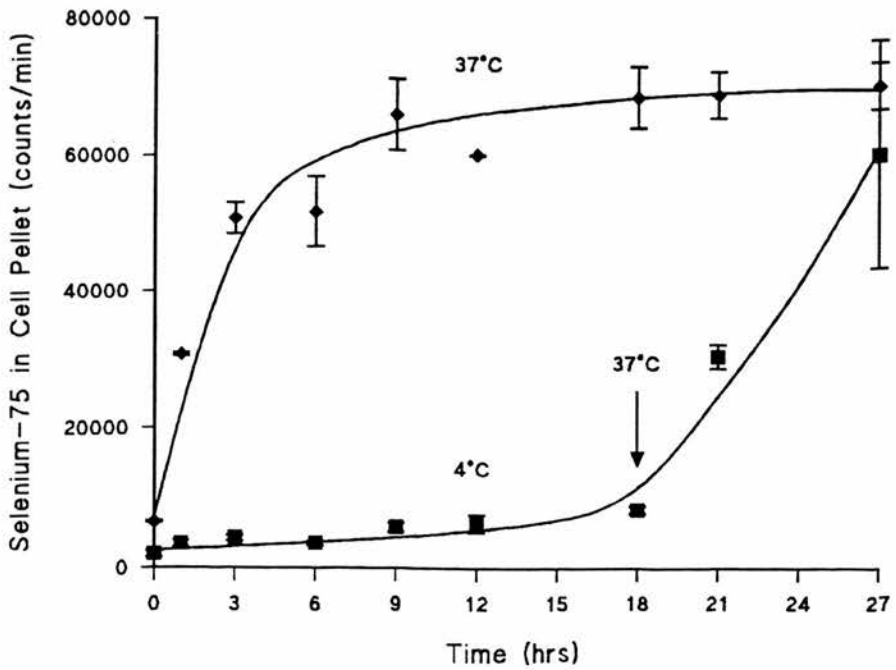
Table 2 :-

	Thyroidal ID-I (fmol/min/mg)	Thyroidal GPx (Units/mg)	Hepatic ID-I (fmol/min/mg)	Hepatic GPx (Units/mg)
Se+ I+	1054 ± 69	0.072 ± 0.0034	3915 ± 105	0.931 ± 0.0638
Se- I+	1539 ± 134	0.035 ± 0.0024	396 ± 34	Not Detectable
Se+ I-	1567 ± 101	0.114 ± 0.0093	3553 ± 151	1.087 ± 0.0630
Se- I-	2712 ± 196	0.055 ± 0.0027	380 ± 26	Not Detectable

Selenium uptake by cultured human thyrocytes in suspension culture

Human thyrocytes were isolated as above and allowed to recover overnight after which time $Na_2(^{75}SeO_3)$ (Amersham, Bucks, U.K.) was added to the cells (74kBq/million cells/ml). The cells were incubated at 37°C and 400μl aliquots were removed at defined times. The samples were centrifuged (3500g, 5 mins) and the pellet washed 3 times with isotonic saline before counting for ^{75}Se. In a further incubation of thyrocytes from the same preparation, cells were incubated for 18h at 4°C after which time a further 9h incubation was performed at 37°C.

Figure 1 shows the temperature dependence of ^{75}Se uptake by the human thyrocytes. Very little ^{75}Se uptake occured at 4°C, but at 37°C the amount of ^{75}Se associated with the cells increased with time until a plateau level was achieved after 12h.



Discussion

These results show that in culture, human thyroidal ID-I expression appears to be dependent on both TSH and selenium supply. However, TSH was able to produce a marked stimulation of ID-I activity in the absence of an extracellular supply of selenium (table 1). In the rat the level of thyroidal ID-I activity appears to be related to the level of plasma TSH (**12**) and not the selenium status of the animal (table 2). We have also shown in the rat that the loss of GPx activity is noticeably less marked in the thyroid than in the liver. These observations are consistent with the view that the thyroid, but not the liver is able to retain selenium in situations where the supply of the trace element is limited. In addition these data are also in keeping with the suggestion of Behne that in selenium deficiency, selenium supply is diverted preferentially to selenoproteins other than GPx (**9**). We have now demonstrated that ID-I is one of these proteins.

The mechanism by which the thyroid retains selenium in situations where the supply of the trace element is insufficient is unclear, but our observation that selenium uptake and retention is temperature dependent (figure 1) suggests that an energy dependent process may be involved.

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Species Differences in Thyroidal Iodothyronine Deiodinase Expression and the Effect of Selenium Deficiency on its Activity

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Thyroxine (T_4) is commonly regarded as a prohormone requiring 5' monodeiodination to produce the biologically-active hormone triiodothyronine (T_3). The monodeiodination of T_4 occurs in most tissues and is catalysed by the iodothyronine deiodinases (ID). Liver, kidney and muscle contain a type I ID (IDI) whilst brain, pituitary and brown adipose tissue express a type II enzyme. IDI is a selenoenzyme and synthesis of the enzyme in liver and kidney does not occur in selenium deficiency (1). In contrast to the type I enzyme, type II ID is not a selenoenzyme but its expression is decreased in selenium deficiency as a consequence of the increased plasma T_4 concentrations (2).

Under normal circumstances more than 80% of plasma T_3 is derived from the action of IDI on T_4 in non-thyroidal tissue and less than 20% of T_3 is derived by *de novo* synthesis in the thyroid. In iodine deficiency and in selenium deficiency (3) the thyroid may provide an important source of plasma T_3. Thyroidal production of T_3 may arise either by *de novo* synthesis on thyroglobulin or by intra-thyroid deiodination of T_4 (rat thyroid expresses high activities of IDI) but the relative quantitative importance of these two pathways of T_3 production is unclear.

We have investigated the role that thyroidal ID may play in T_3 production in rats with normal or low selenium status and in primary culture of sheep and human thyrocytes.

We determine IDI activity in the thyroid and liver obtained from a number of species by using [^{125}I] reverse T_3 as substrate and determining [^{125}I] release (Table 1). Whilst all species studied expressed IDI activity in the liver, the activity was very low or undetectable in goat, cattle, rabbit, sheep, pig, llama and deer with the highest activities being found in mice, rats, guinea pigs and man. These observations were confirmed using affinity labelling experiments with [^{125}I] bromoacetyl reverse T_3 and were carried out as described in (1).

In sheep thyrocytes grown in the presence or absence of inhibitors of IDI (iopanoic acid 10^{-4} M) or *de novo* thyroid hormone synthesis (methimazole 10^{-3} M) T_3 production was unaffected by iopanoic acid addition but was inhibited to 60% of control values. Methimazole had no effect on T_3 production in human thyrocytes whilst iopanoic acid decreased the rate of T_3 production to 27% of control values. These data suggest that in cultured sheep thyrocytes T_3 production arises from *de novo* synthesis on thyroglobulin whilst in human thyrocytes T_3 production arises from deiodination of T_4 within the thyrocyte. Thus we found that when sheep thyrocytes were grown in the presence of Na[^{125}I], ^{125}I incorporation into T_3 and T_4 occurred whilst no [^{125}I] incorporation was found in the human system. We have, as yet, been unable to demonstrate any effect of selenium status on T_3 production in cultured human thyrocytes.

Table 1. Thyroidal IDI Activity in Various Species.

	Deiodinase Activity (fmol I/min/mg protein)
Rat	1296
Guinea Pig	283
Mouse	79
Human	56

Goat, cattle, rabbit, sheep, pig, llama and deer each had activities less than 4 fmol I/min/mg protein.

Table 2. Effect of Se Status on Thyroid IDI and glutathione peroxidase activity.

	Se +	Se –
Deiodinase (fmol I/min/mg protein)	1907 ± 615	1903 ± 501
Glutathione Peroxidase (U/min protein)	0.184 ± 0.0680	0.076 ± 0.0248

Activities are shown as the mean ± SD of rats (fourteen per group) fed a selenium deficient (Se–) or selenium supplemented (Se+) diet for six weeks as described in reference 2.

Selenium deficiency in rats for six weeks from weaning had no significant effect on thyroidal IDI activity (Table 1) whilst in the liver IDI activity was reduced to approximately 5% of values in selenium-sufficient rats. Similarly, selenium deficiency decreased thyroidal glutathione peroxidase activity to approximately 50% of control values (Table 2) whereas in the liver the activity was decreased to < 1% of control values.

Thus there are marked species differences in the expression of thyroidal IDI. Consequently, in species such as the sheep which only poorly express the thyroidal IDI thyroidal deiodination of T_4 cannot play an important role in T_3 production. In man and the rat the activity of thyroidal ID appears largely resistant to changes in selenium status. This ability of the thyroid to maintain the expression of IDI in selenium deficiency allows T_3 to be produced by both *de novo* synthesis and intrathyroidal deiodination of T_4. In addition, the maintenance of glutathione peroxidase activity in the presence of selenium deficiency provides continued protection of the thyroid from the high intracellular levels of hydrogen peroxide which are produced by the gland during hormone synthesis.

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SELENIUM, TYPE I IODOTHYRONINE 5'-DEIODINASE ACTIVITY AND THYROID HORMONE METABOLISM IN THE RAT.

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INTRODUCTION

At least five selenoproteins have been identified and cloned, indicating multiple functions for selenium in the maintenance of normal cell biochemistry (reviewed 1). Mechanisms for the incorporation of selenocysteine into proteins have also been recognised, further supporting the idea of specific biological and biochemical functions for selenium (2). In addition to the functional selenoproteins already identified, there are other selenium-containing proteins with, as yet, no recognised function (3) These proteins have been detected by SDS-polyacrylamide gel electrophoresis, after *in vivo* treatment of rats or mice with ^{75}Se (4,5). The identification of several selenoproteins is consistent with diverse biological functions for the trace element and thus the multiple effects of selenium deficiency in animals and humans.

The ability of the selenium-containing glutathione peroxidases to destroy hydrogen peroxide and lipid peroxides in the cell is essential in the prevention of the formation of potentially injurious-free radicals. Thus the effects of selenium deficiency which are reversed by either selenium or vitamin E supplementation are probably related to the antioxidant functions of the glutathione peroxidases in plasma or cell membranes or cytosol (1,3). Such disorders include skeletal and cardiac muscle myopathies and liver necrosis, which occur in many animal species (6). In selenium deficiency decreases in the glutathione peroxidases at specific sites in the cell might explain some of the diverse responses to the deficiency. However, despite this potential for variable responses, changes in glutathione peroxidase activity cannot explain all the effects of selenium deficiency (7-9). This has provided the impetus for the characterisation of other biochemical functions for selenium in other metabolic processes, and the discovery of an essential role for selenium in maintaining normal thyroid hormone and iodine metabolism (10-13). This function is exerted via control of the deiodinase enzymes which regulate the conversion of thyroxine (T_4) to the

more metabolically active 3,3′,5-triiodothyronine (T_3). Type I iodothyronine 5′-deiodinase (IDI) is a selenoprotein and its expression is regulated by dietary selenium supply (10-15). Although type II iodothyronine 5′-deiodinase (IDII) is not a selenoprotein, its activity is regulated in selenium deficiency by changes in plasma T_4 concentrations (16).

SELENIUM AND THYROID HORMONES

The effects of selenium deficiency on thyroid hormone and iodine metabolism have been reviewed in detail (12,13,17). Figure 1 gives a summary of some of these findings which are derived mainly from experiments with rats. The left side of the

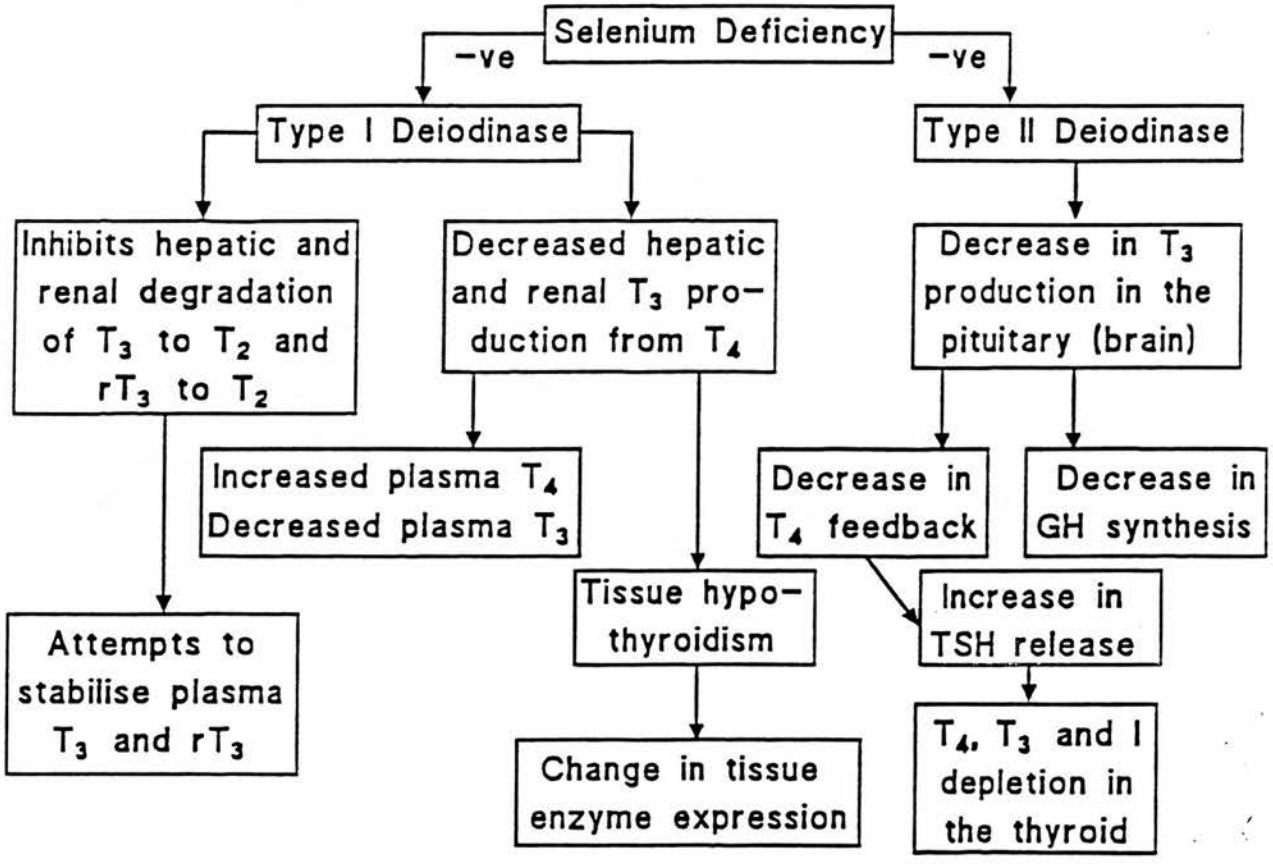

Figure 1: The effects of selenium deficiency on hyroid hormone metabolism.
Key: T_2, diiodothyronine; TSH, thyroid stimulating hormone; rT_3, reverse triiodothyronine; GH, growth hormone; I, iodine.

diagram, shows the effects of selenium deficiency mediated through decreases in IDI activity. There is decreased hepatic and renal T_3 production from T_4, which contributes to increased plasma T_4 and decreased plasma T_3 concentrations, both

characteristic of selenium deficiency. IDI also degrades T_3 to T_2 and reverse T_3 to T_2. This reaction is also inhibited in selenium deficiency which helps to maintain plasma T_3 concentrations (18). Increased T_4 concentrations and decreased T_3 concentrations may cause changes in hepatic enzyme activities which are observed in selenium deficiency, such as the increase glutathione S-transferase levels. However, further research is required to confirm an association between thyroid hormones and these changes. As illustrated at the right-hand side of Figure 1, inhibition of IDII activity decreases T_3 production in the pituitary and the brain. Selenium deficiency also decreases IDII activity in brown adipose tissue (not shown in the diagram). Impaired T_3 production in the pituitary influences the mechanisms which control TSH production and release; thus there are increased plasma TSH concentrations in selenium deficiency. This increase in TSH is consistent with decreased levels of T_4, T_3 and iodine in the thyroid gland of selenium-deficient rats. Additionally, the decrease in T_3 formation may cause lower growth hormone concentrations in the pituitary and thus impaired growth in selenium deficiency.

SELENIUM STATUS AND THYROID HORMONE METABOLISM

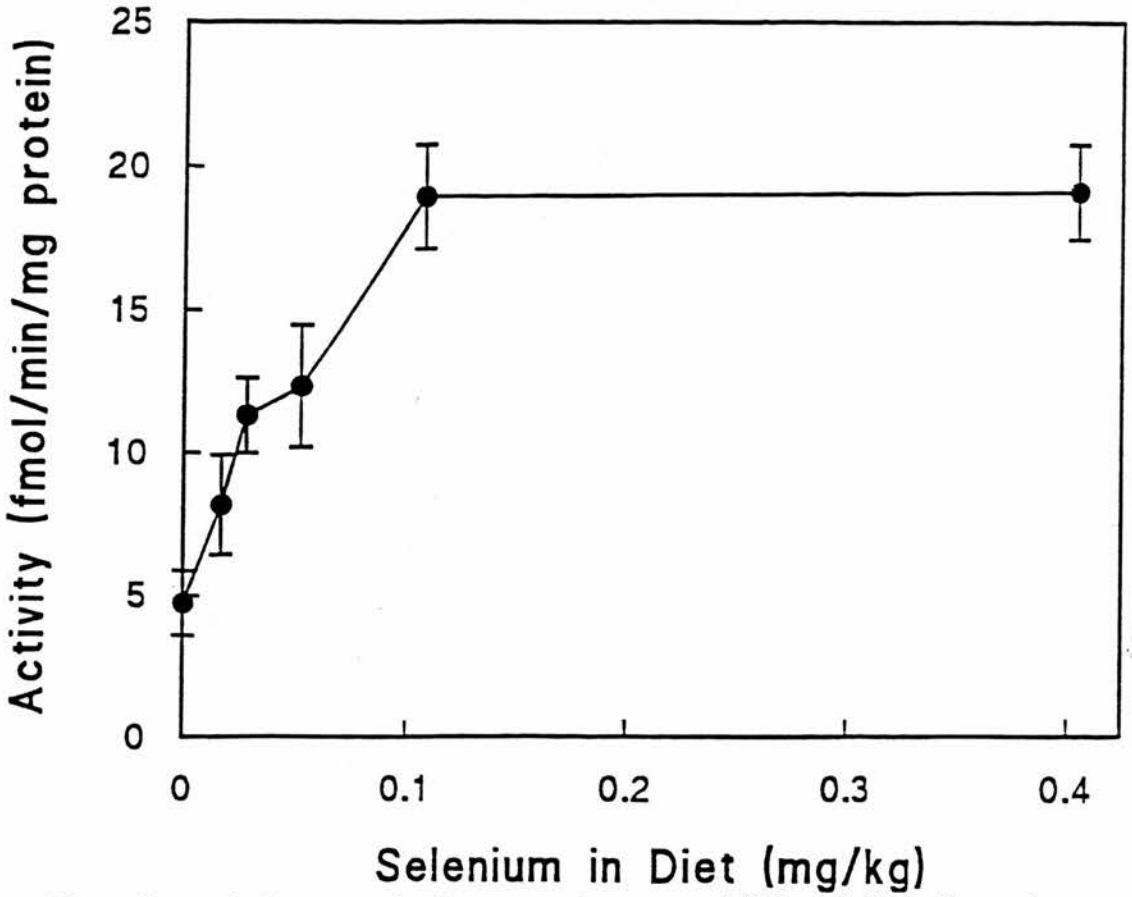

Figure 2: The effect of dietary selenium supply on renal IDI activity in the rat. Animals were fed semisynthetic diets (10,11) for six weeks from weaning. Results are mean ± SEM, 6 animals/ group.

The data summarised in figure I were obtained with rats of a very low selenium status; for example hepatic GSHPx activity was less than 1% of normal values. As this severity of deficiency is rarely encountered in animals or humans, we have also investigated the effects of more "biologically relevant" dietary selenium concentrations on thyroid hormone metabolism. In rats, hepatic IDI activity, determined as deiodination of rT_3, increased with dietary selenium concentration throughout the range from severely deficient (0.005 mg Se/kg diet) to adequate (0.1 mg Se/kg diet). There was no further increase in activity as dietary selenium was increased to 0.4 mg Se/kg. IDI protein, detected by affinity labelling with ^{125}I-bromoacetyl-rT3 or ^{125}I-bromoacetyl-T_4, reflected the changes in IDI activity in response to the different selenium intakes. Additionally, decreasing dietary selenium

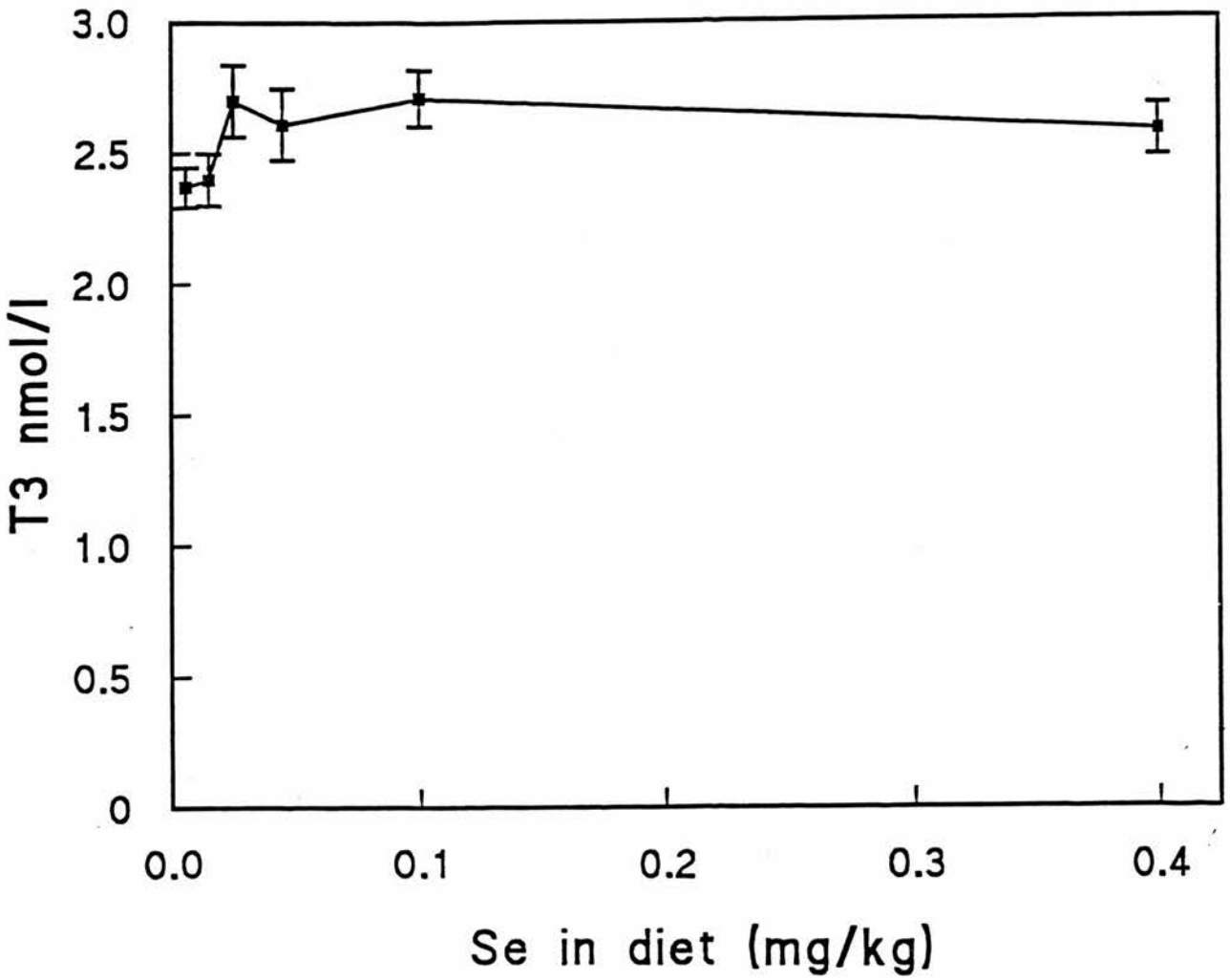

Figure 3: the effect of dietary selenium supply on plasma T_3 concentrations in the rat. Animals were fed semisynthetic diets (10,11) for six weeks from weaning. Results are mean ± SEM, 6 animals/ group.

intake from 0.1 mg/kg diet to 0.005 mg/kg diet caused inhibition of renal IDI activity (Figure 2) and IDII activity in both brain and brown adipose tissue . The decreases in IDI activity caused increases in plasma T_4 concentrations and lower hepatic catabolism of T_3 to T_2 (18 and J.R. Arthur, F. Nicol, B. Gill, Y. Guo and G.J. Beckett, unpublished observations). As well as decreased T_3 catabolism by hepatic IDI, induction of thyroidal IDI also helps to maintain plasma T_3 concentrations (13,14). Thus plasma T3 concentrations are only decreased by 5 to 10% at very low dietary selenium intakes (Figure3; 10,13,14), which is less than would be predicted if the concentrations were only controlled by hepatic or renal IDI activity (see Figure 2)

CONCLUSIONS

Despite compensatory mechanisms which tend to maintain T_3 production, thyroid hormone concentrations are abnormal in plasma and organs of selenium-deficient rats and may contribute to adverse effects of the deficiency, especially when free radical activity is supressed by adequate vitamin E concentrations (12,13,18). As well as in the rat thyroid, significant IDI activity occurs in the thyroids of mice, guinea pigs and humans. Thus all these species have a potential mechanism for maintaining plasma T_3 concentrations in selenium deficiency and in hypothyroid stress (19). However, cattle, sheep, pigs, llamas, goats, deer and rabbits have no significant activity in the gland which cannot therefore provide T_3 by deiodination (19). If no other mechanisms exist to maintain plasma and tissue T_3 concentrations in these latter species, they may be more susceptible to thyroid-related effects of selenium deficiency.

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Effects of combined iodine and selenium deficiency on thyroid hormone metabolism in rats[1,2]

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ABSTRACT This paper compares the effects of combined iodine and selenium deficiency, of single deficiencies of these trace elements, and of no deficiency on thyroid hormone metabolism in rats. In rats deficient in both trace elements, thyroidal triiodothyronine (T_3), thyroidal thyroxin (T_4), thyroidal total iodine, hepatic T_4, and plasma T_4 were significantly lower, and plasma thyroid-stimulating hormone (TSH) and thyroid weight were significantly higher than in rats deficient in iodine alone. Plasma and hepatic T_3 concentrations were similar in the dietary groups. Hepatic type I iodothyronine deiodinase (ID-I) activity was inhibited by selenium deficiency irrespective of the iodine status. Type II deiodinase (ID-II) activity in the brain was significantly higher and in pituitary, significantly lower in combined deficiency than in iodine deficiency alone. These data show that selenium can play an important role in determining the severity of the hypothyroidism associated with iodine deficiency. *Am J Clin Nutr Suppl* 1993;57:240S–3S.

KEY WORDS Selenium deficiency, iodine deficiency, iodothyronine deiodinase, thyroid hormones, glutathione peroxidase

Introduction

Iodine deficiency has profound effects on the mental and physical development of the fetus and the child; severe iodine deficiency may result in cretinism (1). Several adaptive processes can protect people from the harmful effects of iodine deficiency. The trapping of iodide is increased and intrathyroidal iodine metabolism is modified to increase 3,3',5-triiodothyronine (T_3) production at the expense of thyroxin (T_4) synthesis. These changes in thyroid hormone metabolism are thought to be controlled by thyroid-stimulating hormone (TSH), the release of which is triggered by a decrease in plasma T_4. The result of these modifications in thyroid hormone synthesis in iodine deficiency is that although plasma T_4 may fall to subnormal concentrations, plasma T_3 is maintained (2).

Approximately 80% of plasma T_3 is normally derived from 5'-deiodination of T_4 in nonthyroidal tissues such as the liver, kidney, and muscle. These tissues contain a type I iodothyronine deiodinase enzyme (ID-I) that can catalyse both 5- and 5'-monodeiodination of T_4 and other iodothyronines (3). We demonstrated that ID-I is a selenoenzyme (4–7) and Berry et al (8) cloned the enzyme, confirming the presence of selenium as a selenocysteine residue, coded for by a uracil-guanine-adenine (UGA) triplet, at the active site (8). The cloning data and the results of Behne et al show that only one selenium residue is present in the enzyme (9).

The pituitary, central nervous system, brown adipose tissue, and brain contain a type II deiodinase (ID-II) that can perform only 5'-deiodination (3). This enzyme is probably not a selenoenzyme (10) but its expression is suppressed by high plasma concentrations of T_4 such as are found in hyperthyroidism or selenium deficiency (3–5, 10). It has been suggested that selenium deficiency as well as iodine deficiency may be involved in the pathogenesis of endemic cretinism (11, 12), and that selenium deficiency exacerbates some aspects of the hypothyroidism associated with iodine deficiency in rats (13). This paper describes comprehensive studies of the effects of combined iodine and selenium deficiencies on thyroid hormone metabolism in rats.

Methods

Animals and diets

Male, Hooded Lister, weanling rats of the Rowett Institute strain were divided into four groups and offered one of the following diets for 7 wk: *1*) selenium- and iodine-supplemented control (Se+I+), *2*) selenium-deficient (Se−I+), *3*) iodine-deficient (Se+I−), and *4*) selenium- and iodine-deficient (Se−I−). The basal diet (Se−I−) was prepared as described previously with the omission of potassium iodate from the mineral mix (4); it contained < 0.005 mg Se/kg diet and < 0.1 mg I/kg. Where appropriate, the basal diet was supplemented with 0.1 mg Se/kg as selenite and/or 1.0 mg I/kg as potassium iodate. Distilled water and food were available ad libitum; food intake was recorded daily and animals were weighed weekly. At the end of the experiment, rats were anesthetized with diethyl ether, and blood was taken into heparinized tubes by cardiac puncture. Livers were perfused with 0.15 mol KCl/L at 4 °C, removed, and immediately frozen in liquid nitrogen. The thyroid gland, brain, kidney, and brown adipose tissue were also removed and frozen immediately in liquid nitrogen.

Hormone assays

Plasma and organ T_4 and T_3 concentrations were measured by radioimmunoassay (4). Free T_4 was determined using a mi-

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croencapsulated-antibody method (IDS Ltd, Wallsend, Tyne and Wear, UK). Plasma TSH was assayed by radioimmunoassay by using reagents provided by the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD, and the Scottish Antibody Production Unit, Carluke, Lanarkshire, UK.

Deiodinase assays

5′-ID-I activity in liver was measured as the production of T_3 from T_4 as substrate (4). The 5-ID-I activity in liver homogenates was determined as [^{125}I]-diiothyronine production from [^{125}I]T_3 [0.37 MBq (1 μCi); specific activity > 44 TBq/g > 1200 mCi/mg); substrate and product were separated by thin-layer chromatography. The 5′ deiodinase activity in brain, pituitary, and brown adipose tissue was measured by using [^{125}I]-reverse T_3 as substrate (14).

Tissue T_3 and T_4 concentrations

Liver homogenates were extracted with ammoniacal methanol; thereafter, T_3 and T_4 were assayed in the extracts by radioimmunoassay. The T_3, T_4, and iodine contents of rat thyroid were determined as described previously (15).

Glutathione peroxidase

This was measured as described previously (16).

Statistics

Results were analyzed by ANOVA, and the residual standard error was used to calculate the significance of the difference between group-mean values.

Results

There were no significant differences in final body weight among the dietary groups, nor was the food intake different (results not shown). Iodine deficiency produced a significant increase in thyroid size and weight, but the heaviest and largest glands were found in selenium- and iodine-deficient rats (**Table 1**).

Rats receiving either of the selenium-deficient diets had significantly lower hepatic glutathione peroxidase activities than did animals receiving the diets supplemented with selenite. Iodine deficiency had no significant effect on glutathione peroxidase activity (Table 1).

Selenium deficiency alone caused a significant decrease in plasma T_3, but in iodine-deficient or selenium- and iodine-deficient rats, plasma T_3 concentration were not significantly changed.

Plasma total and free T_4 concentrations were increased by selenium deficiency and were decreased by iodine deficiency compared with values in Se^+I^+ rats. The lowest plasma total T_4 concentrations occurred in Se^-I^- rats whereas free T_4 was below the detection limit of the assay in all iodine-deficient rats irrespective of selenium status (Table 1).

Selenium deficiency alone had no significant effect on plasma TSH despite the increase and decrease in plasma T_4 and T_3 concentrations, respectively. Iodine deficiency produced a significant increase in plasma TSH with concentrations $\approx$ 100% greater than that found in control animals. The highest TSH concentrations occurred in the rats deficient in both selenium and iodine; their concentrations were $\approx$ 250% greater than those in rats deficient in iodine alone.

Hepatic 5- and 5′-ID-I activity was inhibited by selenium deficiency irrespective of the iodine status. Iodine deficiency had no effect on hepatic 5′-ID-I activity (**Table 2**). Selenium deficiency significantly decreased ID-II activity in the pituitary. In iodine deficiency, ID-II was increased in the brain but not the pituitary. In selenium-and-iodine-deficient rats, brain ID-II activity increased to significantly higher levels than it did in iodine deficiency, but ID-II activity in the pituitary was decreased (Table 2).

Hepatic T_4 and T_3 concentrations reflected the plasma hormone concentrations. Hepatic T_4 was increased in selenium deficiency and decreased in iodine deficiency. The lowest hepatic T_4 concentrations occurred in combined selenium and iodine deficiency. Hepatic T_3 was slightly decreased in selenium deficiency, but in iodine deficiency and combined selenium and iodine deficiency, hepatic T_3 was slightly increased (Table 1).

TABLE 1
Plasma and tissue hormone concentrations and thyroid weights in selenium and iodine deficiency*

	Se+I+	Se−I+	Se+I−	Se−I−
Plasma				
Total T_4 (nmol/L)	67.20 ± 2.870	88.75 ± 1.658†	39.8 ± 2.16†	33.4 ± 4.77†
Total T_3 (nmol/L)	1.24 ± 0.32	0.96 ± 0.21	1.29 ± 0.18	1.04 ± 0.21
Free T_4 (pmol/L)	2.56 ± 0.52	6.93 ± 0.58†	ND	ND
TSH (μg/L)	0.93 ± 0.11	1.28 ± 0.15‡	2.69 ± 0.40†	4.35 ± 0.52†
Liver				
Total T_4 (ng/g liver)	14.8 ± 1.3	25.7 ± 3.2‡	5.9 ± 0.54§	2.26 ± 0.3†
Total T_3 (ng/g liver)	1.80 ± 0.06	1.40 ± 0.04†	2.1 ± 0.10‡	2.14 ± 0.15‡
Thyroid				
Total T_4 (nmol/g protein)	2545 ± 108	1905 ± 191‡	228 ± 45†	6.7 ± 2.0†
Total T_3 (nmol/g protein)	330 ± 16	243 ± 15‡	56 ± 9.7†	20 ± 3.9†
Total I (μmol/g protein)	58.7 ± 4.9	47.9 ± 3.1‡	6.82 ± 0.82†	0.82 ± 0.28†
Thyroid weight (mg)	21.81 ± 1.37	22.24 ± 0.74	34.85 ± 1.77‡	54.13 ± 41.03‡

* $\bar{x}$ ± SEM. Se+I+, control; Se+I−, iodine deficient; Se−I+, selenium deficient; Se−I−, selenium and iodine deficient; ND, not detected.
†‡§ Significantly different from control: †$P < 0.001$, ‡$P < 0.05$, §$P < 0.01$.

TABLE 2
Type I and type II deiodinase and glutathione peroxidase activity in selenium and iodine deficiency*

	Se+I+	Se−I+	Se+I−	Se−I−
Liver				
5′-ID-I (pmol $T_3 \cdot min^{-1} \cdot g\ protein^{-1}$)	113 ± 7.6	8 ± 0.8†	104 ± 8.4	6 ± 3.1†
5-ID-I (%)‡	20.2 ± 4.4	5.6 ± 2.3§	16.6 ± 2.1	7.8 ± 2.3§
Glutathione peroxidase (U/g protein)	1.44 ± 0.43	0.003 ± 0.002†	1.58 ± 0.13	0.004 ± 0.002†
Pituitary				
ID-II (pmol $T_3 \cdot min^{-1} \cdot g\ protein^{-1}$)	18.04 ± 2.38	10.78 ± 1.38‖	19.22 ± 3.21	13.39 ± 1.37§
Brain				
ID-II (pmol $I^- \cdot min^{-1} \cdot g\ protein^{-1}$)	0.678 ± 0.034	0.498 ± 0.100	1.148 ± 0.172‖	1.614 ± 0.085†

$\bar{x}$ ± SEM. Se+I+: control; Se+I−: iodine deficient; Se−I+: selenium deficient; Se−I−: selenium and iodine deficient.
†§‖ Significantly different from control: †$P < 0.001$, §$P < 0.05$, ‖$P < 0.01$.
‡ T_3-to-T_2 conversion.

In the thyroid of the selenium-deficient rats, there was a significant, ≈ 15% decrease in T_3, T_4, and iodine content. Iodine deficiency caused a > 75% decrease in iodine and hormone concentrations, and combined selenium and iodine deficiency produced the greatest decreases in thyroidal T_4, T_3, and iodine content (Table 1).

Discussion

Concurrent selenium deficiency can exacerbate the goiter and hypothyroidism associated with iodine deficiency (Table 1). Thyroid weight, gland size, and plasma TSH were all significantly increased in rats fed the diet deficient in both trace elements when compared with iodine-deficient rats. Concurrent selenium deficiency also caused a further reduction in thyroidal T_3, T_4, and iodine content and plasma and hepatic T_4, when compared with iodine deficiency ($P < 0.05$).

In both iodine and combined iodine and selenium deficiency, plasma and hepatic T_3 concentrations did not decrease, and in the liver, T_3 concentrations slightly increased. The increases in T_3 were not reflected in hepatic malic enzyme activity, which was decreased in iodine-deficient rats and unchanged in combined selenium and iodine deficiency. The ability of the rats with combined iodine and selenium deficiency to maintain plasma and tissue concentrations of T_3 when hepatic ID-I is markedly inhibited can be explained by compensatory mechanisms. In iodine deficiency, plasma T_3 concentrations are maintained as a result of increased thyroidal T_3 synthesis, driven by increased plasma TSH. It has been suggested that in iodine deficiency, the major part of circulating T_3 is in fact derived from the thyroid (17). Our data support this view because when iodine deficiency is accompanied by selenium deficiency, there is a marked decrease in hepatic conversion of T_4 to T_3 but thyroidal T_4, T_3, and iodine are diminished as a result of the thyroidal hormone synthesis being stimulated by increased TSH. The ability to maintain normal T_3 concentrations in combined selenium and iodine deficiencies would be potentiated by an inhibition of 5-ID-I activity because it is this reaction that is responsible for catabolism of T_3.

Thyroidal ID-I may have a role in generating the increased production of T_3 in conditions that increase plasma TSH (18). Other data supporting this view are elevations of thyroidal ID-I in the groups with elevated TSH (JR Arthur, GJ Beckett, S Beech, F Nicol, and PWH Rae, unpublished observations). This increase in thyroidal ID-I in the rats deficient in both iodine and selenium is of interest because these data suggest that the thyroid gland is able to retain adequate amounts of selenium for ID-I production when the liver cannot achieve this. In selenium-deficient rats, thyroidal glutathione peroxidase activity was decreased by 50% whereas hepatic glutathione peroxidase activity was decreased to < 1% of control values (14).

Although hepatic thyroid hormone status is maintained in combined iodine and selenium deficiency, this is not the case for the pituitary (Table 2) and brown adipose tissue (19). In rats with combined deficiency, plasma TSH was highest as a result of very low plasma total and free T_4 and also of a decrease in pituitary ID-II. Similarly, the decreased concentrations of mitochondrial uncoupling protein in brown adipose tissue caused by combined selenium and iodine deficiency in rats probably results from decreased ID-II activity. Such deficient animals are likely to have a diminished capacity for thermogenesis.

We did not measure an end-organ marker of function in the brain, but in rats with combined selenium and iodine deficiencies there was a large stimulation in brain ID-II activity. This indicates that the brain is under a greater hypothyroid stress than in iodine deficiency alone. The crucial question is whether this increase in cerebral ID-II can compensate for the very low plasma T_4 concentrations of combined selenium and iodine deficiency, particularly because the brain utilizes plasma T_3 very ineffectively. The mechanism behind the differential tissue effects of combined selenium and iodine deficiencies on ID-II remains to be resolved. ID-II is not a selenoenzyme but its expression can be controlled by plasma T_4 concentrations (10). Thus, when plasma T_4 is low, such as in iodine deficiency or combined iodine and selenium deficiency, ID-II would be expected to increase, and indeed this occurs in the brain; in pituitary (Table 2) and brown adipose tissue, however, ID-II decreased.

In conclusion, our data suggest that selenium deficiency can increase the hypothyroid stress associated with iodine deficiency alone. Rats are able to compensate for this in tissues containing ID-I. These tissues utilize plasma T_3, and by increasing thyroidal T_3 production and decreasing peripheral catabolic 5-deiodination, T_3 concentrations are maintained. Pituitary and brown adipose tissue, which contain ID-II and require plasma T_4, cannot compensate for the decreased plasma T_4 that occurs in combined deficiencies. In contrast, in the brain, ID-II is induced by

combined selenium and iodine deficiency, which may ameliorate some of the potentially damaging effects of impaired thyroid hormone metabolism.

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